

This electronic thesis or dissertation has been downloaded from the King's Research Portal at <https://kclpure.kcl.ac.uk/portal/>



Immune Suppression in Multiple Myeloma Strategies to Overcome NK Cell Inhibition

Giunti, Giulia

Awarding institution:
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

END USER LICENCE AGREEMENT



Unless another licence is stated on the immediately following page this work is licensed

under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International

licence. <https://creativecommons.org/licenses/by-nc-nd/4.0/>

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

IMMUNE SUPPRESSION IN MULTIPLE MYELOMA: STRATEGIES TO OVERCOME NK CELL INHIBITION

**A thesis submitted for the degree of
Doctor of Philosophy**

Giulia Giunti, MSc

Department of Haematological Medicine

King's College London

2013

Abstract

Multiple myeloma (MM) is an essentially incurable malignancy associated with profound cellular and soluble immune deficiencies. Despite recent progresses in the treatment of MM, the prognosis remains frequently poor due to the difficulty in targeting MM progenitor cells, which are responsible for disease relapse. Immunotherapy, and in particular the employment of Natural Killer (NK) cells, offers the potential to target and eliminate MM cells within the bone marrow stromal sanctuaries, where they appear to be better protected against conventional therapeutic interventions. However, these strategies have so far provided limited clinical benefit, possibly reflecting the various escape mechanisms employed by MM cells to avoid immune recognition.

The work presented in this thesis aims to further elucidate the mechanisms underlying MM-induced inhibition of NK cells and to investigate the therapeutic potential of immunomodulatory strategies to reverse this inhibition.

Initially, the effect of co-culturing MM cell lines and healthy donor (HD) peripheral blood mononuclear cells (PBMCs) on NK cell phenotype and function was analysed. The results demonstrate that MM cell lines are able to impair NK cell cytolytic activity. This inhibition, which correlates with the downregulation of activating receptors such as NKG2D, NKp30, and DNAM-1, was shown to be the product of direct and contact-dependent interactions between MM and NK cells, without the need for other peripheral blood components. Importantly, the analysis of NK cells isolated from MM patients show that they display the same suppressed phenotype and activity as healthy donor NK cells co-cultured in the presence of MM cells lines, thereby suggesting that the detected suppression of NK cell activation by MM cells is a function of MM cells rather than any inherent defect in NK cells isolated from MM patients.

In the light of this immunosuppressive effect, MM cells genetically-modified with a self-inactivating lentiviral vector encoding CD80 (B7.1) and IL-2 were tested for their ability to enhance and recover NK cell functional competence. Our results show that the *in vitro* co-culture of healthy donor or MM patient PBMCs with CD80/IL-2-modified MM cells is able to expand NK and T cell numbers, and to induce a significant increase in the fraction of NK cells expressing activating receptors such as NKp44, NKG2D, NKp30, and CD69, when compared to unmodified MM cells. More importantly for potential therapeutic applications, stimulated NK cells from healthy donors show increased cytolytic activity. These data suggest that the stimulation of PBMCs with CD80/IL-2-modified MM cells may be able to overcome the immune suppressive functions of unmodified MM cells and to stimulate NK and T cell mediated responses.

Therefore, vaccination with CD80/IL-2-modified MM cells may represent a potential strategy for NK cell recovery and stimulation and, possibly, for the induction of a broad ranging immunological responses against multiple myeloma cells.

Declaration of Originality

The work presented in this thesis was performed by Giulia Giunti, all else has been appropriately referenced.

Table of contents

Abstract	2
Declaration of Originality	4
Table of contents	5
Table of Figures	10
Summary of Tables	14
Acknowledgments	15
Presentations at National and International Conferences	17
Abbreviations	18
Chapter 1 Introduction.....	22
1.1 Multiple Myeloma.....	22
1.1.1 Introduction and pathogenesis	22
1.1.2 Diagnosis and standard treatment.....	24
1.2 The immune system and Natural Killer (NK) cells.....	27
1.2.1 Soluble immunity: cytokines, chemokines and antibodies	27
1.2.2 Cellular immunity	28
1.2.2.1 Natural Killer (NK) cells	29
1.2.2.1.1 Human NK cell subsets	30
1.2.2.1.2 NK cell activation and regulation	32
1.2.2.1.3 Human Natural Killer Receptors (NCR).....	34
The Killer-Cell Immunoglobulin-like Receptors (KIR)	35
Receptors mediating Natural Cytolysis	36
Activating co-receptor molecules	38
Regulation of NCR expression.....	40
1.2.2.1.4 The cross-talk between NK cells and dendritic cells (DCs) in the initiation of the immune response	41
NK maturation induced by DC	42
DC maturation induced by NK cells	43

Role of NK–DC interactions in T and NK cell responses.....	44
Role of NK–DC crosstalk in anti-tumour responses	45
1.3 Immunodeficiencies in multiple myeloma (MM)	47
1.3.1 Cytokine-mediated immunosuppression	48
1.3.2 Cellular-mediated immunosuppression	49
1.3.3 Cellular immune defects in MM	50
1.4 Immunotherapy for MM	52
1.4.1 Humoral immunotherapy	54
1.4.1.1 Thalidomide and immunomodulatory drugs (IMiDs)	54
1.4.1.2 Monoclonal antibodies	55
1.4.2 Cellular immunotherapy.....	56
1.4.2.1 NK cells	56
1.4.2.2 T cells	57
1.4.2.3 DC-loaded with myeloma-associated antigens.....	57
1.4.2.3.1 Idiotypic (Id) proteins.....	58
1.4.2.3.2 Tumour-associated antigens	59
1.4.2.4 Whole MM cells	61
1.4.2.4.1 DC loaded/fused with MM cells	61
1.4.2.4.2 Whole cancer cell vaccines	65
Genetic modification of tumour cells	71
1.4.3 Considerations and future directions	74
1.4.4 Aims of the thesis	75
Chapter 2 Materials and Methods.....	76
2.1 Cell culture.....	76
2.1.1 Preparation of media and solutions	76
2.1.2 Cell lines	77
2.1.2.1 K562.....	77
2.1.2.2 MM cell lines	78
2.1.2.3 293T cells.....	79
2.1.2.3.1 BL-15 cells	79
2.1.2.4 Mycoplasma test by polymerase chain reaction (PCR)	80
2.1.2.5 Agarose gel preparation and electrophoresis	81

2.1.3 Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs).....	82
2.1.3.1 Isolation of PBMCs.....	82
2.1.3.2 Selection of cryopreserved MM patients PBMCs	83
2.1.4 Primary MM cells	85
2.1.4.1 Cryopreservation and thawing of PBMCs and CD138 ⁺ cells	85
2.2 Lentiviral vectors.....	86
2.2.1 Construction of lentiviral vectors.....	86
2.2.2 Lentivirus production.....	87
2.2.2.1 Plasmid expansion and purification	87
2.2.2.1.1 E. coli transformation.....	87
2.2.2.1.2 Mini prep	88
2.2.2.1.3 DNA purity and concentration evaluation	88
2.2.2.1.4 Diagnostic restriction digestion.....	89
2.2.2.1.5 Maxi prep	91
2.2.2.2 Lentivirus production in helper cells	92
2.2.2.2.1 Lentivirus production and concentration using 293T cells	92
2.2.2.2.2 Lentivirus production and concentration using BL-15 cells (Nesbeth <i>et al.</i> 2006)	93
2.2.2.3 Target cell transduction.....	94
2.2.2.3.1 Lentivirus titration	94
2.2.2.3.2 Lentivirus infection of target cells	94
2.2.2.3.3 Target cells biotinylation and treatment with PEI	95
2.3 Co-cultures	96
2.3.1 Co-cultures of PBMCs or NK cells with MM cell lines	96
2.3.2 Co-culture of PBMCs with unmodified or genetically-modified MM cells	96
2.3.3 Culture of PBMCs in the presence of immunomodulatory drugs (IMiDs).....	97
2.4 Cell sorting.....	98
2.4.1 Magnetic cell sorting	98
2.4.2 Fluorescence activated cell sorting (FACS)	100
2.5 Flow cytometry	102
2.5.1 Quality control of flow cytometric assays (Mahnke and Roederer 2007)	102

2.5.2 Staining of cell lines and primary MM plasma cells	103
2.5.3 Staining of PBMCs and NK cells	104
2.5.3.1 PBMCs	104
2.5.3.2 NK cells and T cells.....	105
2.5.4 Detection of NK cell cytotoxicity.....	107
2.5.4.1 Non-radioactive killing assay	107
2.5.4.2 NK cell degranulation and IFN- γ production	108
2.6 ELISA	113
2.7 MTT assay.....	115
2.8 Statistical analysis	116
Chapter 3 Results	117
3.1 Multiple myeloma-mediated inhibition of NK cells.....	117
3.1.1 Introduction	117
3.1.2 Results	119
3.1.2.1 MM cell lines inhibit NK cell activity	119
3.1.2.2 The MM-induced inhibition of NK cells activity is not caused by the exhaustion of NK cells	122
3.1.2.3 The MM cell line U266 is able to inhibit NK cell activity through contact-dependent interactions	123
3.1.2.4 MM cell-induced impairment of NK cells is associated with decreased expression of the activating receptors NKG2D, NKp30 and DNAM-1.....	124
3.1.2.5 Primary NK cells isolated from MM patients display impaired cytolytic activity and an immune phenotype similar to healthy donor NK cells co-cultured with MM cell lines..	126
3.1.2.6 MM-induced impairment of NK cell activity does not depend on the presence of other peripheral blood mononuclear cells	128
3.1.3 Discussion.....	130
3.2 The production of high virus titers for MM cell genetic modification	132
3.2.1 Introduction	132
3.2.2 Results	134
3.2.2.1 Construction and production of lentiviral vectors	134
3.2.2.2 Optimisation of a protocol to transduce the MM cell line U266.....	137
3.2.2.3 Generation of genetically-modified U266 cells for functional assays	149

3.2.2.4 Characterisation of genetically-modified U266 cells	151
3.2.2.5 Genetic modification of CD138 ⁺ MM plasma cells	156
3.2.3 Discussion.....	158
3.3 Enhanced immune responses to stimulation with IL-2/CD80 genetically-modified cells ...	162
3.3.1 Introduction	162
3.3.2 Results	165
3.3.2.1 Set-up of optimal conditions for co-culture of PBMCs and genetically-modified MM cells	165
3.3.2.2 Allogeneic stimulation of healthy donor (HD) PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the <i>in vitro</i> expansion and activation of NK cells	169
3.3.2.3 Allogeneic stimulation of HD PBMCs with IL-2/CD80 genetically-modified U266 MM cells induces the <i>in vitro</i> expansion and activation of T cells	173
3.3.2.4 IMiDs (Pomalidomide, Lenalidomide) fails to show synergy with IL-2/CD80 genetically-modified U266 MM cells for the induction of NK cell cytolytic activity in Healthy Donors.....	175
3.3.2.5 Allogeneic stimulation of MM patient PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the <i>in vitro</i> expansion of NK cells	178
3.3.2.6 Allogeneic stimulation of MM patient PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the <i>in vitro</i> expansion of T cells.....	185
3.3.3 Discussion.....	187
Chapter 4 Concluding remarks	191
Bibliography	194

Table of Figures

Figure 1-1 Myeloma initiation in the context of B cell development (Morgan <i>et al.</i> 2012)	23
Figure 1-2 Treatment approaches in MM.....	25
Figure 1-3 The biological function of NK cells (Vivier <i>et al.</i> 2008).	30
Figure 1-4 Human NK cell subsets (Cooper <i>et al.</i> 2001)	30
Figure 1-5 The dynamic regulation of NK cell effector functions (Vivier <i>et al.</i> 2011).	33
Figure 1-6 NK cell receptors (Vivier <i>et al.</i> 2011).	35
Figure 1-7 Interactions between NK cells and DCs in the periphery (Cooper <i>et al.</i> 2004).	43
Figure 1-8 Interaction between NK cells, DCs and T cells in the lymph node (LN) (Cooper <i>et al.</i> 2004)	45
Figure 1-9 Role of DC cross-talk in the immune response against tumours (Degli-Esposti and Smyth 2005).....	46
Figure 1-10 Interaction between MM cells and the immune system.	48
Figure 1-11 Suggested DC-based vaccine strategies for patients with MM (Nguyen-Pham <i>et al.</i> 2012).	64
Figure 2-1 Schematic maps of the transfer vectors (Chan <i>et al.</i> 2005a)	87
Figure 2-2 Restriction map of pLV.IL-2/CD80.....	90
Figure 2-3 Detection of viral particles in first and second harvest following transfection of 293T cells.	93
Figure 2-4 Purity of CD138 ⁺ cells before and after magnetic cell sorting.	99
Figure 2-5 Purity of CD20 ⁺ B cells before and after magnetic cell sorting.....	99
Figure 2-6 Purity of CD3 ⁻ CD56 ⁺ NK cells before and after magnetic cell sorting.	100
Figure 2-7 Optimisation of CD107a/intracellular IFN- γ detection assay.	110
Figure 2-8 ELISA standard curve fit.....	114
Figure 3-1 Impaired cytolytic activity of NK cells following co-culture with MM cell lines.	120
Figure 3-2 Effect of MM cell lines on the expression of the activating NK receptor NKG2D.	121
Figure 3-3 Effect of incubation time on the expression of NKG2D on NK cells following incubation with the MM cell line U266.	122
Figure 3-4 Granzyme B release from PBMCs cultured either in complete medium alone or in the presence of the MM cell line U266 or K562 cells.	123

Figure 3-5 Contact-dependent inhibition of NK cells following co-culture with U266 MM cell line.	124
Figure 3-6 Effect of MM cell line U266 on the expression of activating NK receptors.	125
Figure 3-7 Impairment of NK cell activity by MM in healthy donor and MM patients.	126
Figure 3-8 Effect of the MM cell line U266 on the expression of activating NK receptors.	127
Figure 3-9 Effect of peripheral blood cell components on the MM cell-induced functional inhibition of NK cells.	128
Figure 3-10 Effect of peripheral blood mononuclear cells on the MM cell-induced functional inhibition of NK cells.	129
Figure 3-11 Effect of peripheral blood mononuclear cells on MM cell-induced downregulation of NKG2D.	129
Figure 3-12 Gel electrophoresis pattern of plasmid restriction fragments.	135
Figure 3-13 Lentiviral vectors titration in human erythroleukemic cell line K562.	135
Figure 3-14 IL-2 production following infection with lentiviral vectors.	136
Figure 3-15 Comparison between K562 and U266 cell permissivity to LV.IL-2/CD80.	137
Figure 3-16 Effect of lentiviral transduction on cell viability.	138
Figure 3-17 Impact of infection enhancers on U266 cell permissivity and viability.	139
Figure 3-18 Impact of infection enhancers on U266 cell proliferation and viability.	139
Figure 3-19 Effect of different infection enhancers on U266 cell transduction.	140
Figure 3-20 Comparison between K562 and U266 cell permissivity to LV.IL-2/CD80-conjugated with PMP and evaluation of lentiviral concentration efficiency.	142
Figure 3-21 Effect of lentiviral transduction with PMP-conjugated virus on cell viability.	143
Figure 3-22 Biotinilation of U266 cells.	144
Figure 3-23 Effect of PEI treatment and target cells biotinitation on the percentage of genetically-modified cells following infection with LV.IL-2/CD80.	145
Figure 3-24 Lentiviral vector titration in K562 and U266 cell lines.	146
Figure 3-25 CD80 expression levels following infection with lentiviral vectors.	146
Figure 3-26 Stability of the CD80 ^{high} and CD80 ^{dim} peaks over time.	147
Figure 3-27 CD80 expression levels in RPMI8226 cells following infection with lentiviral vecors.	147
Figure 3-28 FSC and SSC characterisation of CD80 ^{high} and CD80 ^{dim} peaks.	148
Figure 3-29 CD80 expression levels pre and post-fluorescence-activated cell sorting.	149

Figure 3-30 IL-2 production following infection with lentiviral vectors.....	150
Figure 3-31 Comparison and normalisation of IL-2 secretion levels for LV.IL-2 and LV.IL-2/CD80 modified U266 cells.....	151
Figure 3-32 Effect of lentiviral transduction on cell viability.	152
Figure 3-33 Stability of CD80 expression levels after fluorescence-activated cell sorting of CD80 positive cells.....	153
Figure 3-34 IL-2 production levels from uninfected or infected U266 cells on day 1 and day 30 post-sorting.	154
Figure 3-35 Evaluation of PD-L1 expression on U266 MM cell line and healthy donor PBMCs before and after treatment with IFN- γ	155
Figure 3-36 CD28 expression levels on U266 cells following transduction with LV.CD80 or LV.IL- 2/CD80.....	155
Figure 3-37 CD80 expression levels following infection of MM primary cells with the lentiviral vector IL-2/CD80.....	156
Figure 3-38 IL-2 production by LV.IL-2/CD80 CD138 ⁺ primary MM cells following infection with lentiviral vectors.	157
Figure 3-39 Effect of lentiviral transduction primary MM cells viability.	157
Figure 3-40 LV.IL-2/CD80 U266 cells viability after irradiation.	165
Figure 3-41 CD80 expression of LV.-modified and uninfected cells after irradiation.	166
Figure 3-42 IL-2 production after irradiation by LV.IL-2/CD80 U266 cells.....	166
Figure 3-43 Effect of <i>in vitro</i> co-culture of healthy donor PBMCs with unmodified U266 (right column) or IL-2/CD80 cells (left column) on NK cell numbers and phenotype.....	168
Figure 3-44 Effect of <i>in vitro</i> co-culture of HD PBMCs with un-modified of modified U266 cells on NK cell numbers.....	169
Figure 3-45 fect of <i>in vitro</i> co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on NCR expression.....	171
Figure 3-46 Effect of <i>in vitro</i> co-culture of healthy donor (HD) PBMCs with un-modified of modified U266 cells on NK cell cytolytic activity towards unmodified U266.....	172
Figure 3-47 Effect of <i>in vitro</i> co-culture of healthy donor (HD) PBMCs with un-modified of modified U266 cells on T cell numbers.	173
Figure 3-48 Effect of <i>in vitro</i> co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on CD8 ⁺ T cells HLA-DR and CD69 expression.....	174

Figure 3-49 Effect of <i>in vitro</i> co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against U266 cells.	175
Figure 3-50 Effect of <i>in vitro</i> co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against K562 and U266 cells.	176
Figure 3-51 Effect of <i>in vitro</i> co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against U266 MM cells.	176
Figure 3-52 Effect of <i>in vitro</i> co-culture of healthy donor PBMCs with un-modified or modified U266 cells on NK cell cytolytic activity against unmodified U266.	177
Figure 3-53 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified of modified U266 cells on NK cell numbers.	179
Figure 3-54 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified or modified U266 cells on NCR expression.	181
Figure 3-55 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified or modified U266 cells on NCR expression levels.	182
Figure 3-56 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified of modified U266 cells on NK cell cytolytic activity towards unmodified U266 cells.	183
Figure 3-57 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified or modified U266 cells on NK cell degranulation and IFN- γ secretion levels.	184
Figure 3-58 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified of modified U266 cells on T cell numbers.	185
Figure 3-59 Effect of <i>in vitro</i> co-culture of MM patient PBMCs with un-modified of modified U266 cells on CD8 ⁺ T cell receptor expression.	186

Summary of Tables

Table 1-1 Principal TAAs employed in MM-immunotherapy and stimulatory potential, adapted, expanded and integrated from (Zhang <i>et al.</i> 2012)	60
Table 1-2 Different anti-tumour vaccine strategies and related advantages and disadvantages (Harrison and Cook 2005)	63
Table 1-3 Summary of studies investigating CD80-expressing cancer vaccines.	69
Table 2-1 Characteristics of K562 cells	77
Table 2-2 Characteristics of MM cell lines (Drexler and Matsuo 2000)	78
Table 2-3 Phenotypic characteristics of MM cells.....	78
Table 2-4 Mycoplasma PCR reaction mix.....	80
Table 2-5 Mycoplasma PCR cycle.....	81
Table 2-6 Characteristics of MM patients	84
Table 2-7 Spectrometric profile of IL-2/CD80 plasmid DNA	89
Table 2-8 Diagnostic restriction digestion mix	91
Table 2-9 Transfection reaction mix.....	92
Table 2-10 Magnetic cell sorting strategy	99
Table 2-11 MM cells antibody stain mix.....	104
Table 2-12 PBMCs proliferation antibody stain mix.	105
Table 2-13 NK cell activation antibody stain mix.	106
Table 2-14 T cell activation antibody stain mix.	106
Table 2-15 CD107a-IFN- γ antibody staining mix	112

Acknowledgments

At the end of a journey, you can never help but to look back and smile with gratitude at every person who shared it with you and made it possible.

Starting from the very beginning of this path, I would like to express my deepest gratitude to Professor Schey, Professor Farzaneh and the Cancer Vaccine Institute for believing in me and giving me one of the greatest opportunities of my life.

This PhD has also been one of the greatest challenges of my life. I learned more about myself in these four years than in my whole life, most of the time the hard way, but I confronted and overcame my limitations. I hope that the results of this thesis might contribute to our understanding of the interactions between the immune system and cancer and, hopefully, advance cancer immunotherapy. My ambition is to continue on this path and to be involved in the translation of these discoveries from bench to bedside.

My journey would have been extremely difficult without the support of very talented scientist like Sharham, Lucas, Dave, Linda and Yolanda, who have always provided me with great ideas and suggestions.

During these years I have met lots of different people and everyone, in his own way, has inspired me to become a better scientist and a better person. In particular, I would like to thank you to my Rayne family: Annie, Sabine, David, Thomas, Mary, YuQian, Sneha, Cinzia, Pilar, Rebecca, Wendy and many more. You have been a constant source of laughs, support and, maybe a little bit too often, of junk food, and I am grateful to have you in my life.

However, despite this fantastic team and my hard work, I would not have got here if it was not for the love and support I receive from my family. Babbo, Mamma, Laura and Massimo: this thesis is dedicated to you.

“Nobody said it would be easy, they just promised it would be worth it.”

Adapted from Harvey MacKay

Presentations at National and International Conferences

Giulia Giunti, Lucas Chan, David Darling, Sharham Kordasti, Linda Barber, David Malone, Farzin Farzaneh and Steve Schey (2012) "CD80/IL-2 mediated inhibition of multiple myeloma induced suppression of NK cell activity" *Presented at the 20th European Society of Cell and Gene Therapy Conference 2012, Versailles, France.*

Giulia Giunti, David Malone, Lucas Chan, David Darling, Linda Barber, Sharham Kordasti, Farzin Farzaneh and Steve Schey (2011) "CD80/IL2 expressing myeloma cells for immune gene therapy of multiple myeloma" *Presented at the 52nd British Society for Haematology Conference 2012, Glasgow, United Kingdom.*

Giulia Giunti, David Malone, Oppenheim David, Lucas Chan, David Darling, Linda Barber, Sharham Kordasti, Farzin Farzaneh and Steve Schey (2010) "CD80/IL2 expressing myeloma cells for immune gene therapy of multiple myeloma" *Presented at the 13th International Myeloma Workshop 2010, Paris, France.*

Abbreviations

°C	Degrees Celsius
7-AAD	7-Aminoactinomycin D
Abs	Antibodies
ADCC	Antibody Dependent Cytotoxicity
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
APC	Antigen Presenting Cell
ASCT	Autologous Stem Cell Transplantation
ATCC	American Type Culture Collection
BCG	Bacillus Calmette-Guérin
CD	Cluster of Differentiation
CDCC	Complement-Dependent Cellular Cytotoxicity
CLL	Chronic Lymphocytic Leukaemia
CTL	Cytotoxic T cell
CTLA-4	CTLA-associated antigen 4
COX	Cyclooxygenase
CR	Complete Response
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic Cell
DEAE	Diethylaminoethyl cellulose
DLI	Donor Lymphocyte Infusion
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNAM-1	DNAX Accessory Molecule 1

DPBS	Dulbecco's Phosphate-Buffered Saline
DTH	Delayed-Type Hypersensitivity
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunosorbent spot (assay)
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft Versus Host Disease
GVM	Graft Versus Myeloma
HBSS	Hank's Balanced Salt Solution
HD	Healthy Donor
HGF	Hepatocytes Growth Factor
HIV	Human Immuno-deficiency Virus
HLA	Human Leukocyte Antigen
HSCs	Haematopoietic Stem Cells
HSCT	Haematopoietic Stem Cell Transplantation
hTERT	Human Telomerase Reverse Transcriptase
JAK	Janus Kinase
KIR	Killer-cells Immunoglobulin-like Receptor
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin Growth Factor
IL	Interleukin
ILCs	Innate Lymphoid Cells
IMiDs	Immunomodulatory Drugs
IRES	Internal ribosome entry sites
ITAM	Immuno-receptor Tyrosine-Based Activation Motif
ITIM	Immuno-receptor Tyrosine-Based Inhibition Motif
IU	Infective Units

KIR	Killer Immunoglobulin-like Receptor
LFA-1	Lymphocyte Function Associated Antigen-1
LN	Lymph nodes
LTR	Long Terminal Repeat
LV	Lentivirus
mAbs	Monoclonal Antibodies
MAIT	Mucosal-Associated Invariant T (cells)
MFI	Mean Fluorescence Intensity
MGUS	Monoclonal Gammopathy of Undetermined Significance
MICA/B	Major histocompatibility complex class I-related chains <i>A/B</i>
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
MMP	Matrix Metalloproteases
NCR	Natural Cytotoxicity Receptor
NF- κ B	Nuclear Factor-kappa B
NK cell	Natural Killer Cell
NKT	Natural Killer T Cell
NKR	NK cell receptor
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed Cell Death-1
PD-1L	Programmed Cell Death-1 Ligand
PEI	Polyethylenimine
PCR	Polymerase Chain Reaction
PMP	Para Magnetic Particle
RIC	Reduced-Intensity Conditioning
RNA	Ribonucleic Acid
RPMI1640	Roswell Park Memorial Institute medium 1640
STAT	Signal Transducers and Activators of Transcription
TAA	Tumour-Associated Antigen

TCR	T Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumour necrosis factor
TLRs	Toll-Like Receptors
Th	T helper
Treg	T Regulatory cells (regulatory T cells)
VEGF	Vascular Endothelial Growth Factor
VSV-G	Vesicular Stomatitis Virus G glycoprotein
WHO	World Health Organisation
WPRE	Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element
WT-1	Wilms' Tumour-1

Chapter 1 Introduction

1.1 Multiple Myeloma

1.1.1 Introduction and pathogenesis

Multiple myeloma (MM) is a clonal B cell disorder in which malignant plasma cells expand and accumulate in the bone marrow leading to cytopenia, bone resorption, and production of the characteristic monoclonal protein (Fonseca *et al.* 2009).

It commonly presents with bone pain, tiredness, lethargy and renal impairment (Palumbo and Anderson 2011). It accounts for approximately 1% of neoplastic diseases and 13% of haematological cancers (Palumbo and Anderson 2011).

MM is a disease of the elderly, with the median age at diagnosis of approximately 73 years, and appears to occur more and more frequently in younger individuals (Phekoo *et al.* 2004). Although the majority of cases present *de novo* with evidences of advancing disease, it is now recognised that myeloma is preceded by a period of asymptomatic monoclonal gammopathy of undetermined significance (MGUS) phase in virtually all patients (Bird *et al.* 2011), with a cumulative risk of overall progression of 1-2% per annum (Kyle and Rajkumar 2007). Whilst survival has significantly improved in the last 15 years, myeloma remains the paradigm of an incurable disease.

As a tumour of antibody-producing plasma B cells, it is instructive to understand how B cells develop, in order to appreciate how myeloma initiates and progresses (Figure 1-1). B cells are a class of lymphocytes involved in the humoral branch of the adaptive immune response. Their main role is the secretion of proteins called antibodies (see 1.2.1) in the body fluids.

The initiation of the myeloma tumour cell results from the physiological gene rearrangement process necessary to generate antibody diversity within B cells which, combining with genetic changes, including translocations, copy number abnormalities, mutation, hyper-methylation, and micro-DNA abnormalities, leads to the immortalisation of a myeloma-propagating cell (Gonzalez *et al.* 2007).

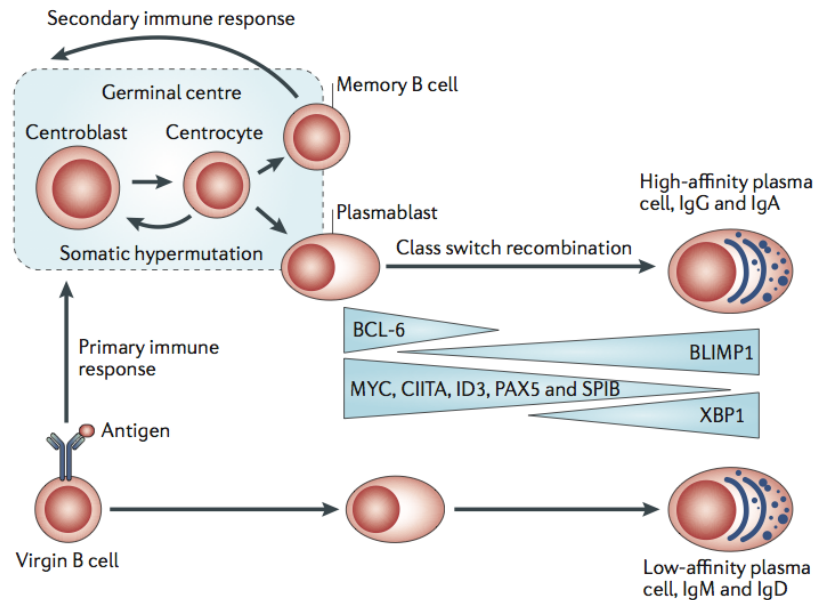


Figure 1-1 Myeloma initiation in the context of B cell development (Morgan *et al.* 2012)

Subsequently, the malignant plasma cell migrates from the germinal centre to the bone marrow, where it continues to evolve and proliferate. At this stage, the interactions and establishment of a positive feedback loop with the supportive bone marrow microenvironment is crucial for the survival and resistance of myeloma to various treatments (Mitsiades *et al.* 2006; Hideshima *et al.* 2007). The consequent disruption of the bone marrow microenvironment affects the homeostasis of the normal plasma cell niche leading to the development of immune suppression. Therefore, it seems that the price of generating a wide antibody repertoire, which is necessary for an effective immune system, is a background rate of B cell tumours and myeloma, particularly later in life. This could explain why clonal expansion of plasma cells in the form of MGUS is often observed in individuals over the age of 60 (Morgan *et al.* 2012).

1.1.2 Diagnosis and standard treatment

The diagnosis of myeloma is based on the presence of at least 10% clonal bone marrow plasma cells, lytic bone lesions and the presence of monoclonal proteins in the serum or urine (Palumbo and Anderson 2011). The recommended tests for diagnosis include physical examination, routine laboratory testing, bone marrow examination and skeletal imaging. Myeloma patients bearing chromosomal abnormalities such as the translocations t(11;14), t(4;14), t(14;16), t(6;14), t(14;20), hyperdiploidy, and deletion of 17p, have normally a less favourable outcome compared to those showing a normal karyotype (Table 1-1) (Palumbo and Anderson 2011).

Table 1-1 Risk stratification analysis on the basis of genetic abnormalities in MM. Adapted from (Rajkumar 2011).

Category	Characteristics	Frequency
Low-risk	Absence of intermediate-risk or high risk factors	75%
Intermediate-risk	t(4;14) and absence of 17p deletion or high-risk gene-expression-profiling signature	10%
High risk	Presence of 17p deletion or high-risk gene-expression-profiling signature	15%

Whilst manifesting clinically as clonal proliferation of plasma cells, the International Myeloma Working Group reports that multiple myeloma is, actually, a heterogeneous disease in terms of prognosis, with some patients dying within a few weeks of diagnosis, whereas others live for longer than 10 years (Fonseca *et al.* 2009). The reason for this heterogeneity is the result of interactions between host factors, such as performance status, comorbidities and age, as well as features intrinsic to the disease biology, such as genetic features of the tumour cells and interactions with the tumour and bone marrow microenvironment.

Treatment strategies include induction regimens, which aim to induce high rates of complete response, followed by maintenance treatment, which aims to delay tumour re-growth. Host factors, in particular age and co-existing conditions, normally influence the choice of the induction regimen because of the relative risk of toxicity from different anti-myeloma agents. Current data support the initiation of induction therapy with

thalidomide (Thalidomid), lenalidomide (Revlimid) or bortezomib (Velcade) in conjunction with autologous haematopoietic stem-cell transplantation for patients under the age of 65 years who do not have substantial heart, lung, renal or liver dysfunction. Reduced intensity conditioning regimens are instead considered for older patients or those with co-existing conditions (Figure 1-2) (Palumbo and Anderson 2011).

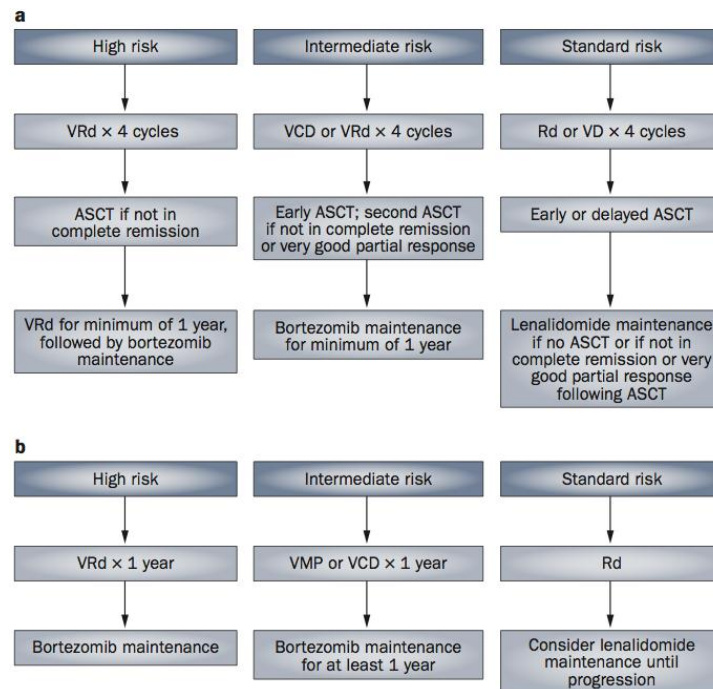


Figure 1-2 Treatment approaches in MM

a) Treatment approaches for newly diagnosed transplant candidates b) Treatment approaches for newly diagnosed elderly, non-transplant candidates. Abbreviations: ASCT, autologous stem cell transplantation; VCD, velcade–cyclophosphamide–dexamethasone; VMP, velcade–melphalan–prednisone; VRd, velcade–lenalidomide–dexamethasone (Rajkumar 2011).

The treatment of multiple myeloma has changed and evolved dramatically in the past decade thanks to the discovery of the central role of the bone marrow microenvironment in myeloma pathogenesis (Caligaris-Cappio *et al.* 1992). This has been the major contributor to the development of new active agents and drug combinations (Podar *et al.* 2009; Palumbo and Anderson 2011). However, the fact that only a fraction of cases shows responses to single agents suggests that the molecular subtype of the disease may influence the response to specific therapies.

Recent studies have highlighted how molecular events that characterise plasma cell transformation are acquired through a branching non-linear pathway rather than in a linear fashion (Morgan *et al.* 2012). The resulting intra-clonal diversity within the MM cell

population has important implications as specific treatments may allow for the selection and expansion of resistant sub-clones, particularly in the bone marrow niches.

Whilst new targeted strategies and drug agents have been demonstrated to prolong progression free survival, benefits in terms of overall survival are still not clear and multiple myeloma remains an incurable disease. New therapeutic approaches are therefore needed and, in this context, serological and cellular approaches that use a different mechanism of action compared to current therapeutic strategies, have the potential to target drug-resistant myeloma sub-clones in bone marrow niches. However, as immune function remains depressed following high-dose therapy for many months, *ex-vivo* or *in-vivo* immunomodulatory strategies to enhance host immunity against myeloma are being currently explored (see Chapter 1.4).

1.2 The immune system and Natural Killer (NK) cells

The human immune system can be divided into a cellular and a soluble molecular arm. With respect to the tremendous knowledge of the immune system that has been developed and broadened over the years, this thesis will not aim to discuss all its components but will cover aspects that relate to this study and, specifically, to the role of NK cells in the control of MM.

1.2.1 Soluble immunity: cytokines, chemokines and antibodies

Cytokines are polypeptides involved in the regulation of cellular activation, differentiation, proliferation, and survival (Murphy 2011). They act by inducing intracellular activation signals through specific cell surface receptors, which are selectively expressed by different subsets of immune cells.

The cytokines that play a major role in the immune response can be categorised as pro-inflammatory cytokines and inhibitory cytokines.

Examples of pro-inflammatory cytokines are the interleukins (ILs), the tumour necrosis factors (TNFs) and the interferons (IFNs).

IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 are type I cytokines belonging to the common cytokine receptor γ -chain family, which all share the same IL-2R γ subdomain (Murphy 2011). Amongst them, of particular importance is IL-2, which was identified for its fundamental role in enhancing NK and T cell proliferation and activity (Cooper *et al.* 2001; Boyman and Sprent 2012). Due to its ability to trigger tumour cell lysis by immune cells (Lotze *et al.* 1981; North *et al.* 2007) this cytokine was administered to patients affected by various malignancies (Rosenberg *et al.* 1985; Lotze and Rosenberg 1986; Rosenberg *et al.* 1987), with a number of complete and partial remissions being reported (Harrison and Cook 2005).

IL-12, IL-15 and IL-18 are, instead, important players in the cross-talk between NK and dendritic cells (DCs) that initiates the adaptive immune response (Walzer *et al.* 2005). IFN- γ belongs to the type II interferon group. It is primarily released from activated DCs, NK and T cells (Ye *et al.* 1995) and is involved in T cell priming (Das *et al.* 2001).

In contrast to the immune stimulatory cytokines, IL-10 and tumour growth factor (TGF)- β

mediate the inhibition of immune responses and modulate the expression of immune receptors (Pratt *et al.* 2007).

Chemokines are a family of small cytokines that control chemotaxis, a process through which resting and activated cells migrate towards specific sites in the body. This represents a critical step, as the convergence of different players of the immune system is required to allow the exchange of signals necessary for the initiation of the immune response. Chemokines exert their biological effects via G protein-linked transmembrane receptors that are selectively expressed by subsets of immune cells (Watson 2002). Within these, CCL19 and CCL21 are particularly important as they recruit CCR7-expressing immune cells to lymph nodes, whereas the chemokine ligand CX3CL1, which is primarily expressed by activated endothelial cells, promotes strong adhesion of leukocytes to activated endothelial cells (Bazan *et al.* 1997; Imai *et al.* 1997).

Antibodies are large proteins produced by B cells. They have the ability to recognise and bind a unique part of a target, called antigen, allowing its neutralisation or its attack by the immune system. This occurs either by activating Fc receptors expressed on Natural Killer (NK) cells or macrophages and leading to antibody-dependent cell-mediated cytotoxicity (ADCC), or by activating the complement cascade, resulting in complement-dependent cytotoxicity (CDCC) (Murphy 2011).

1.2.2 Cellular immunity

The immune system is composed of many different cell types that have distinct functions and distribution pathways in the body. Immune cells arise from haematopoietic stem cells (HSCs) in the liver, thymus and the yolk sac during foetal life, and in the bone marrow after birth (Dzierzak 1999). They are continuously renewed and enter the circulation where they stay or are induced to migrate to specific tissue sites.

The cellular immune system can be divided into the innate and adaptive arm. The innate system includes granulocytes, monocytes, macrophages, dendritic cells (DCs), Natural Killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, and innate lymphoid cells (ILCs) such as NK cells. These cells have the ability to recognise foreign antigens, without the need for prior sensitisation, through germline-encoded

receptors. Once activated, DCs and NK cells develop a crosstalk network of cytokines and chemokines, which then help in shaping the subsequent onset of the adaptive responses (Walzer *et al.* 2005). In fact, activated DCs that have recognised and engulfed pathogens, migrate to the draining lymph nodes where they present digested epitopes of the pathogen to T cells, initiating the adaptive immune response.

Specific T cell subsets, upon recognition of the presented epitopes, proliferate and migrate to the site of infection where they kill the infected cells. This process also generates epitope-specific memory T cells that can protect the host upon re-infection. The inflammatory reaction is terminated by the activation of biochemical programmes with lipid mediators, which enable inflamed tissues to return to homeostasis (Murphy 2011).

This process is controlled by regulatory T cells (Treg), which have the ability to suppress the action of immune cells by secreting inhibitory cytokines (like IL-10 and TGF- β). Tregs can also induce apoptosis or exert direct cytotoxicity on the immune cells (Vignali *et al.* 2008).

In the following sections the main features and characteristics of NK cells, in relation to DCs and T cell activation, will be discussed.

1.2.2.1 Natural Killer (NK) cells

NK cells were serendipitously identified in the early 70s as a disturbing background observed while investigating specific cytotoxic effects of lymphocytes against tumour cell lines. In 1975, they were defined as lymphocytes capable of killing tumour cells in the absence of specific immunisation (Herberman *et al.* 1975). Their presence in non-human mammals and in other vertebrates suggests their evolutionary selection and importance in higher eukaryotes (Vivier *et al.* 2011).

In addition to their direct involvement in the killing of virally-infected and tumour cells, the ability of NK cells to localise to the sites of infection, as well in the major lymphoid organs, installs them in the centre of a complex immunological cross-talk and highlights their important regulatory role in the onset of the adaptive immune response (Brilot *et al.* 2008) (Fig. 1-3).

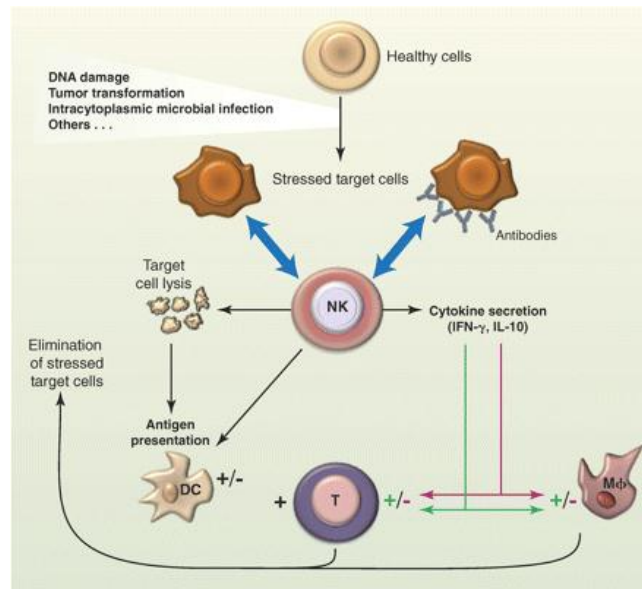


Figure 1-3 The biological function of NK cells (Vivier *et al.* 2008).

1.2.2.1.1 Human NK cell subsets

Human NK cells account for about 10 to 15% of all peripheral blood lymphocytes and are normally defined phenotypically by their expression of CD56 and lack of expression of CD3. With the advent of monoclonal antibodies (Abs), two distinct populations of human NK cells were identified, according to their cell-surface density of CD56 and CD16 (Fig. 1-4) (Cooper *et al.* 2001).

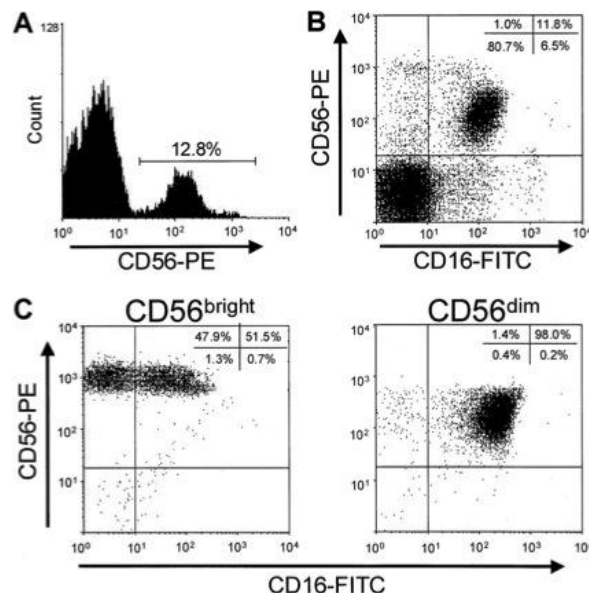


Figure 1-4 Human NK cell subsets (Cooper *et al.* 2001)
Flow cytometric analysis of CD56-PE (A) and CD56-PE/CD16-FITC expression before (B) and after (C) FACS purification of a representative donor.

The CD56 antigen is an isoform of the human neural cell adhesion molecule. Its function in NK cells is still unknown, although early studies suggested that this molecule might mediate interactions between NK cells and target cells (Nitta *et al.* 1989).

CD16 is a low-affinity FcγRIII receptor present on the surface of some NK cells. It has been shown to bind to antibody-coated targets and to direct antibody-dependent cellular cytotoxicity (ADCC) (Leibson 1997).

The majority (about 90%) of human NK cells in the peripheral blood exhibit low-density expression of CD56 (CD56^{dim}) and express high levels of CD16, whereas the remaining 10% is CD56^{bright}CD16^{dim} or CD56^{bright}CD16^{negative} (Cooper *et al.* 2001).

CD56^{dim} NK cells

Early studies on resting CD56^{dim} NK cells have revealed that these cells are more cytotoxic than CD56^{bright} NK cells, although after activation with IL-2 or IL-12 *in vitro*, or following low dose therapy with IL-2, CD56^{bright} and CD56^{dim} cells have similar levels of cytotoxicity (Robertson *et al.* 1992). Consistent with differences in their resting cytotoxic potential, CD56^{dim} NK cells store higher levels of cytotoxic granules compared to CD56^{bright} cells, although they present similar levels of expression of perforin. CD56^{dim} NK cells also seem to be the major subset responsible for ADCC. This particular function, in fact, requires the engagement and activation of CD16 FcγR by antibody-coated targets and therefore, CD56^{dim} NK cells, displaying higher levels of expression of CD16, exhibit greater levels of ADCC compared with the CD56^{bright} subset (Cooper *et al.* 2001).

CD56^{bright} NK cells

Freshly isolated CD56^{bright} human NK cells have been defined as the primary source of NK cell-derived immunoregulatory cytokines, including IFN-γ, tumour necrosis factor β (TNF-β, lymphotoxin), IL-10, IL-13 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Cooper *et al.* 2001). This subset appears to be more responsive to IL-2, due to its constitutive expression of the high-affinity heterotrimeric IL-2 receptor (IL-2Rαβγ). They also seem to be readily responsive to chemotactic signals as they express CCR7, the CXC-chemokine receptor 3 (CXCR3) and the adhesion

molecule L-selectin (CD62L). Consequently, this subset has the potential to traffic to secondary lymphoid organs (Campbell *et al.* 2001b).

These results, taken together with the ability of the CD56^{bright} NK-cell to respond to IL-2 produced by T cells and DCs (Baume *et al.* 1992), postulate a role for this subject in the activation of a cytokine feedback loop between the innate and adaptive immunity within secondary lymphoid organs.

1.2.2.1.2 NK cell activation and regulation

NK cells use preformed lytic granules to facilitate the rapid killing of target cells. Therefore, the cytotoxic potential of NK cells requires a well-regulated mechanism of control.

The “missing self hypothesis” was proposed by Ljunggren and Kärre (Ljunggren and Karre 1986; Ljunggren and Karre 1990) to explain the ability of NK cells to spare normal tissues, whilst killing transformed cells. This hypothesis postulates that NK cell-mediated surveillance depends on the expression of sufficient levels of “self” molecules, which protect target cells from NK cell-mediated lysis. This phenomenon is regulated by the expression of MHC class I–specific inhibitory receptors that functionally dominate over the triggering potential induced by activating receptors. The functions of NK cells, including their cytolytic activity, are therefore regulated by the balance of interactions between a complex array of inhibitory and activating receptors and ligands (Biaassoni 2009) (Figure 1-5).

Any condition altering the strength of the inhibitory receptors/MHC class-I-induced signalling results in direct killing of the infected or transformed cell, as well as in the release of cytokines.

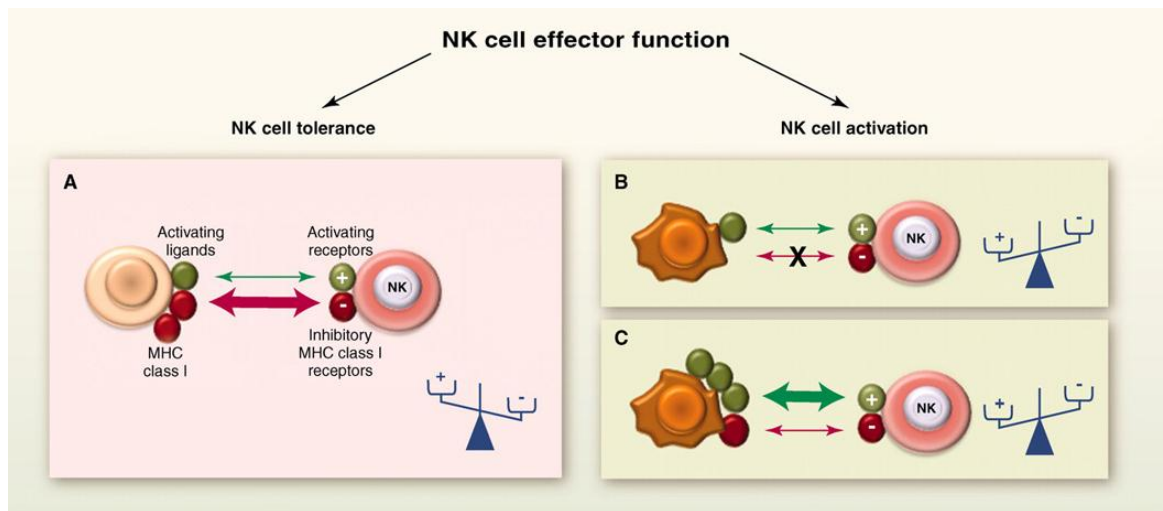


Figure 1-5 The dynamic regulation of NK cell effector functions (Vivier *et al.* 2011).

The study of NK cell-mediated tumour killing provided new insights into the process of NK cell activation. In fact, it has been demonstrated that presence or absence of NK cell ligands on tumour cells alone is inadequate for triggering lysis by resting NK cells, which requires some additional form of stimulation. This priming signal may be nonspecific (e.g. IL-2 or IFN- γ) or can be provided by a tumour cell, while the triggering signal should be specific to prevent auto-reactivity (North *et al.* 2007). This led to the hypothesis that NK-sensitive tumour cells provide both priming and triggering signals. In contrast, NK-resistant tumours evade NK-mediated lysis through the lack of priming and/or triggering ligands for NK cells (Bryceson *et al.* 2011).

NK cells, like T and B cells, have the potential for autoreactivity. This is because the array of receptors that individual NK cells come to express during development is largely random and the MHC ligands recognised by these receptors are inherited independently of the genes encoding for the receptors (Parham 2005). To avoid this risk, an “education system” exists whereby the potentially autoreactive NK cells acquire a state of hypo-responsiveness to stimulation (Fig. 1-6). This is achieved through the induction of an anergic state, as happens in autoreactive T cells and B cells, or through the failure of these NK cells to undergo terminal functional maturation.

Several findings also suggested that the responsiveness of mature NK cells is not fixed but may adapt to a changing environment *in vivo* (Vivier *et al.* 2011). Thus, persistent

stimulation without inhibition results in NK cell hypo-responsiveness, whereas persistent stimulation coupled with commensurate inhibition results in NK cell responsiveness.

Immunological memory is a hallmark of adaptive immunity and is characterised by the long-term persistence of memory cells that rapidly undergo clonal expansion and present enhanced effector functions in response to secondary challenge (Vivier *et al.* 2011). Cooper and colleagues (Cooper *et al.* 2009) recently showed that, once activated, mature NK cells might acquire stable and heritable properties that influence their behaviour during subsequent infections. *In vivo* models showed, in fact, that activated NK cells, after transfer into naïve hosts, return to a quiescent state whilst retaining an intrinsic capacity to respond more robustly after reactivation with cytokines or via engagement of activating NK receptors (Cooper *et al.* 2009).

1.2.2.1.3 Human Natural Killer Receptors (NCR)

NK cell activation is regulated by the integration and amplification of multiple signalling pathways, which depend on a number of surface receptors that bind specific ligands expressed by target cells (Fig. 1-6). Transmission of the signal requires the co-aggregation with other activating/inhibitory receptors at the immuno-synapse interface. This allows NK cells to focus their inhibitory/activating signals to a limited cell surface area while maintaining their potential surveillance and cytolytic capacity in different membrane areas (Bianchini 2009).

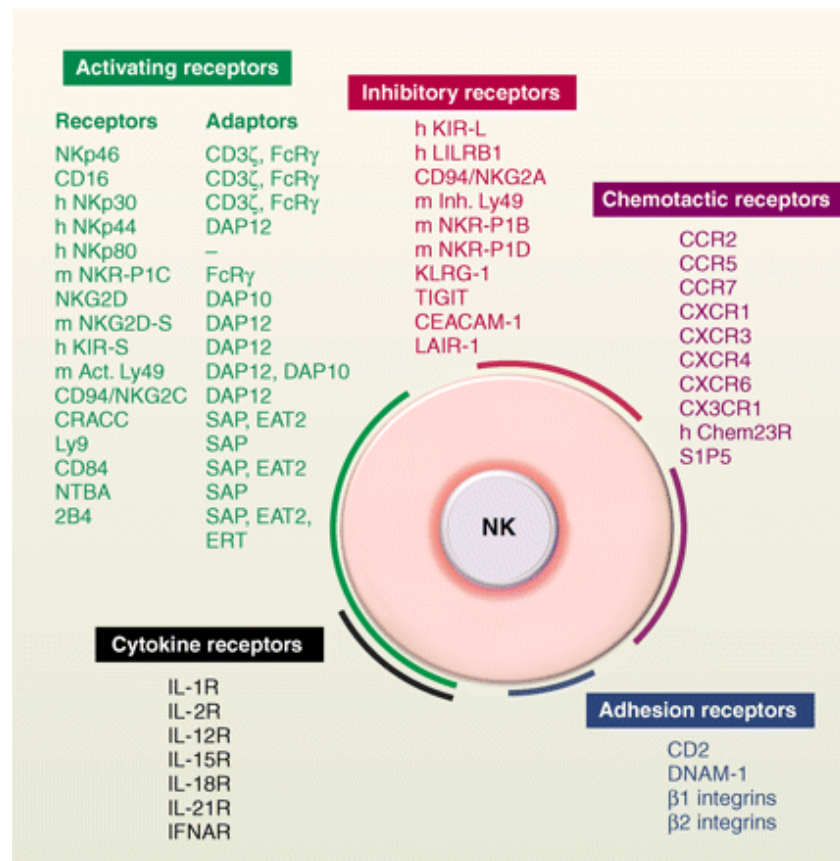


Figure 1-6 NK cell receptors (Vivier *et al.* 2011).

The Killer-Cell Immunoglobulin-like Receptors (KIR)

The KIRs recognise HLA class-I molecules through their extracellular domain. These molecules are normally expressed on most healthy cells in the body, but may be lost upon viral or malignant transformation and during tumour evolution (Beersma *et al.* 1993; Hill *et al.* 1994; Seliger 2008).

Individuals differ in the number and type of KIRs expressed. The variegated expression pattern of KIRs on NK cells may also be explained by the fact that specific KIR gene products are expressed randomly in distinct subsets of NK cells (Valiante *et al.* 1997; Andersson *et al.* 2009). Two major and divergent KIR haplotypes have been identified amongst the human population, which are composed of combinations of both activating and inhibitory KIRs.

Inhibitory and activating KIRs share the same structural features of their extracellular domain (2D or 3D, reflecting the number of Ig-like domains), but have

different cytoplasmic tails with either a long (L) or a short (S) tail, mediating inhibition and activation, respectively (Bashirova *et al.* 2006).

Most functionally mature NK cells express at least one inhibitory receptor specific for a self HLA class-I ligand. In humans, CD94/NKG2A represents the main HLA class-I-specific KIR, as it recognises HLA-A, HLA-B, and HLA-C alleles and HLA-E molecules (Moretta and Moretta 2004; Lanier 2005). Under normal conditions, inhibition signals dominate over activation signals in NK cells (Long 2008). However in some situations, the activation signals may override the inhibitory signals as demonstrated for NKG2D-mediated killing of some MHC class I expressing tumour cell lines in mice (Diefenbach *et al.* 2000; Cerwenka *et al.* 2001).

Receptors with a short tail are, instead, characterised by activating function (KIR2DS, KIR3DS) and their transmembrane portion displays the immune-receptor tyrosine-based activation motif (ITAM), which is responsible for the triggering function of these receptor (Biassoni 2009).

Receptors mediating Natural Cytolysis

The major family of NK cell receptors responsible for the induction of NK-mediated killing is formed by the natural cytotoxicity receptors (NCRs) or natural killer receptors (NKR) NKp46 (NCR1), NKp30 (NCR3), and NKp44, which belong to the Ig-like superfamily, and the NKG2D (KLK1) lectin-like receptor (Biassoni 2008).

Studies conducted to date have demonstrated that, unlike their inhibitory counterparts, these receptors are organised into multichain complexes where the ligand-binding and signal-transducing subunits are separate polypeptides (Yokoyama *et al.* 2004). In this way, their activation results in the engagement of multiple downstream triggering pathways, whose functional cross-talk and integration determine the degree of cell activation.

The role of these receptors in NK cell-mediated target killing has been widely demonstrated in solid tumours (Balsamo *et al.* 2009; Garcia-Iglesias *et al.* 2009; Pietra *et al.* 2012) and haematological malignancies (Sanchez *et al.* 2011), including MM (El-Sherbiny *et al.* 2007; von Lilienfeld-Toal *et al.* 2010). The presence and importance of

these receptors was demonstrated with the aid of anti-NCR monoclonal antibodies and thanks to the identification of NCR-ligands on tumour cells (Pessino *et al.* 1998; Vitale *et al.* 1998; Pende *et al.* 1999; Sivori *et al.* 1999).

NKp46 (NCR1, CD335)

NKp46 is expressed on the surface of either resting or activated NK cells and is thought to be the major receptor responsible for the efficient NK cell-mediated killing of different cancer cell lines (Elboim *et al.* 2010). It is involved, together with NKp44, in the response against influenza virus and in the recognition of tumour cells, such as MM (El-Sherbiny *et al.* 2007), which express the membrane-associated heparan sulfate proteoglycans (Bloushtain *et al.* 2004). NKp46 also helps modulate the adaptive immune response by synergising with NKp30 and DNAM-1 to activate the killing of immature DCs (Wai *et al.* 2011).

NKp44 (NCR2, CD336)

NKp44 is the only receptor expressed by activated NK and $\gamma\delta$ T cells, but not by resting NK cells (Cantoni *et al.* 1999). NKp44 uses a different activating downstream pathway from that used by NKp46. This may explain why activated NK cells display a higher cytolytic potential; in fact, by expressing an additional triggering receptor, the combined signals activated by the engagement of NKp44 and NKp46 may lead to a stronger activation (Biassoni 2009). A possible role for NKp44 in tumour immune-surveillance has been postulated following studies showing its downregulation in the presence of tumour cells such as melanoma (Pietra *et al.* 2012).

NKp30 (NCR3, CD337)

NKp30 is expressed on both resting and activated NK cells. Its role is more complex than initially thought, since it has been involved not only in the response to tumour cells (El-Sherbiny *et al.* 2007; Pietra *et al.* 2012) and pathogens, but also in the modulation and editing of the intensity and quality of the adaptive immune responses. NKp30 has been shown to participate in the cross-talk between NK cells and DCs, particularly in NK-cell mediated killing (Ferlazzo *et al.* 2002; Vitale *et al.* 2005; Pietra *et al.* 2012) and maturation of immature DCs. The mechanism controlling the dual roles of

NKp30 is not clear, although the ratio of NK cells to DCs is thought to be an important factor (Wai *et al.* 2011)

NKG2D (CD314, KLRK1)

NKG2D is constitutively expressed on the surface of all human NK cells, and γ/δ and CD8⁺ T lymphocytes (Biassoni 2009). Human NKG2D has been shown to bind different ligands, which are expressed on the cell surface in response to cellular stresses. In particular, NKG2D interacts with the stress-inducible MHC class I-related molecules MICA and MICB and is involved in the rejection of both virally infected and tumour cells (Raulet 2003; Eagle and Trowsdale 2007; Guerra *et al.* 2008). In addition, Poggi's data indicate that NKG2D may be involved in autoimmunity (Poggi and Zocchi 2007).

NKG2D also binds to surface structures encoded by the homologous genes of the murine retinoic acid-inducible molecules encoded in the *Raet1* gene cluster. A splice variant of the *Raet1G* gene, which encodes a soluble ligand form, may compete with and disrupt the NKG2D/ligand interaction and affect the killing of tumour cells, unveiling an additional escape mechanism for tumour cells from the immune system (Burgess *et al.* 2008). Additionally, other soluble (Pietra *et al.* 2012) and non-soluble (von Lilienfeld-Toal *et al.* 2010) factors have been shown to play a role in tumour-mediated downregulation of NKG2D.

Activating co-receptor molecules

Additional surface receptors are involved in triggering NK cell-mediated cytolytic activity. Some of these molecules are not exclusively expressed by NK cells and they mainly operate as co-receptors or mediate NK cell-to-target cells adhesion (Biassoni 2009). The most important ones include NKp80 (KLRF1), signalling lymphocytic activation molecule or SLAM (2B4, NTB-A, and CS1) receptors, CD16, CD28 and DNAM-1 (CD226).

DNAM-1 is particularly relevant, as it has been implicated in cell adhesion and in triggering of T and NK cell-mediated cytotoxicity. Its function is dependent on the physical association with lymphocyte-associated antigen-1 (LFA-1; CD18/CD11a) (Shibuya *et al.*

1999). Two ligands for DNAM-1 have been identified: CD155 (PVR) and CD112 (Nectin-2) (Bottino *et al.* 2003). The former appears to have a predominant role in inducing DNAM-1 activation. There are some indications that DNAM-1 may also cooperate synergistically with other NCRs and, in particular, NKG2D to trigger NK cell mediated cytotoxicity (Bryceson *et al.* 2006). This combination seems to be important in the protection from tumour development (Iguchi-Manaka *et al.* 2008). Furthermore, DNAM-1 has been found to be an additional pathway used by NK cells in the cross-talk with DCs as these latter cells express DNAM-1 ligands (Iguchi-Manaka *et al.* 2008).

Another important activating receptor is CD16 (FcγRIIIa), which is expressed by the majority of human peripheral blood NK cells, as well as by activated monocytes and T cells. Its binding to the constant region (Fc) of IgG results in phosphorylation of tyrosine residues in the CD3ζ and FcεRIγ ITAM sequences, which then initiate the activating signalling cascade that leads to antibody-dependent cytotoxicity (ADCC) (Bianconi 2009).

The 2B4 (CD244) receptor is expressed on the majority of human NK cells. It binds to CD48, which is commonly expressed by most hematopoietic cells (Korinek *et al.* 1991). Interactions between 2B4 and its ligand results in the induction of proximal activating signals but the magnitude of the signal is not sufficient to induce effective NK cell activation alone (Bryceson *et al.* 2006).

CD28 is a member of the immunoglobulin superfamily and is constitutively expressed on T cells (Schmidt-Weber *et al.* 2002). Its binding to CD80 and CD86, which are found on antigen presenting cells, provides a potent co-stimulatory signal which, in synergy with the T-cell receptor (TCR) signal, promotes T cell activation and survival (Manickasingham *et al.* 1998). Although its expression and importance on murine NK cells has been established (Geldhof *et al.* 1995; Chambers *et al.* 1996), its role in human NK cell activation remains controversial (Galea-Lauri *et al.* 1999; Wilson *et al.* 1999). Firstly, the detection of CD28 on the surface of NK cells is still the object of debate. Galea-Lauri and colleagues (Galea-Lauri *et al.* 1999) have demonstrated that, despite the presence of steady-state levels of CD28 mRNA in NK cells, the detection of this

molecule on their surface strongly varies according to the antibody clone used and also between NK cell lines and individuals. This result could be potentially due to post-translational modification or the generation of splice variants of CD28 (Galea-Lauri *et al.* 1999), which could mask epitopes recognised by a particular antibody clone. Alternatively, the existence of an unknown receptor on NK cells able to interact with CD80 has also been hypothesised (Wilson *et al.* 1999).

Another issue that needs to be addressed is the role of this receptor in NK cell activation in humans, as some studies have demonstrated that CD80 stimulation does not always lead to NK cell activation (Galea-Lauri *et al.* 1999; Wilson *et al.* 1999; Luque *et al.* 2000). Further experiments are needed to demonstrate whether this is the result of the lack of appropriate expression of adhesion and/or co-stimulatory molecules, which may operate in parallel with CD80 to trigger NK cell activation. Additionally, although previous works have shown direct activation of NK cells by CD80-expressing tumour cells (Galea-Lauri *et al.* 1999; Wilson *et al.* 1999; Hardwick *et al.* 2010), including myeloma (Geldhof *et al.* 1995; Yeh *et al.* 1995), further studies should to investigate the ability of NK cells to recognise these molecules and to exclude the possible contribution of T cells (Goodier and Londei 2004) to this phenomenon.

Regulation of NCR expression

Recent studies have demonstrated that NCR expression is dynamic and can be altered by several mechanisms such as cytokines, soluble ligands or through direct contact with targets expressing ligands for NCRs. It is known, in fact, that IL-2 increases the expression of NKG2D (de Rham *et al.* 2007), and stimulates NK cells to express NKp44 (Cantoni *et al.* 1999), whereas TGF- β is able to down-regulate NKG2D and IL-21 affects both NKG2D and NKp44 expression (Castriconi *et al.* 2003; Burgess *et al.* 2006; de Rham *et al.* 2007; Ghio *et al.* 2009).

NCR expression may also be modulated by interactions with their cognate ligands, as exemplified by trogocytosis, where NCRs, such as NKG2D and DNAM-1, were shown to be literally ripped from the NK cell surface or internalised after

receptor-ligand interaction (Groh *et al.* 2002; Mota *et al.* 2004; Coudert *et al.* 2005; Oppenheim *et al.* 2005; Roda-Navarro *et al.* 2006).

1.2.2.1.4 The cross-talk between NK cells and dendritic cells (DCs) in the initiation of the immune response

Recent findings have highlighted the role of NK cells as an evolutionary “bridge” between innate and adaptive immunity. Indeed NK cells, as the centre of a complex crosstalk between DCs and T cells, can influence the outcome of both “specific” adaptive-like responses, as well as “non- specific” innate-like responses (Brilot *et al.* 2008).

DCs are a sparsely distributed migratory groups of bone-marrow-derived leukocytes, which were initially identified due to their particular morphology and functional specialisation for antigen uptake, transport, processing and presentation to T cells (Walzer *et al.* 2005). At the ‘immature’ stage of development, DCs continuously sample the antigenic environment in peripheral tissues and migrate at a slow rate to lymph nodes. Under these steady-state conditions, DCs express low levels of MHC and co-stimulatory molecules, and their interaction with naive T cells leads to T-cell tolerance. By contrast, the encounter with microbial products or tissue damage in the periphery initiates DCs maturation and their rapid migration to lymph nodes. This activation program is, in part, due to the engagement of a complex set of receptors, such as the toll-like receptors (TLRs), which are able to recognise molecules or molecular patterns shared by various classes of microbes. Mature DCs start expressing high levels of MHC and co-stimulatory molecules, such as CD80 and CD86, which allow the activation of naive T cells and secrete an array of cytokines known to enhance NK cell cytotoxic function and IFN- γ production (Walzer *et al.* 2005).

However, this system is more complex than previously thought as DC activation leads to bi-directional and complex interactions among DCs, NK, and T cells, which are responsible for tuning the outcome of the overall immune response.

NK maturation induced by DC

DCs are able to produce a wide range of cytokines that trigger NK cell activation processes such as IFN- γ production, cytotoxicity, and proliferation.

IL-12 is produced by many DC subsets in response to various stimuli, and is known to synergise with IL-18 to induce IFN- γ secretion and NK-cell cytotoxicity (Ferlazzo *et al.* 2004).

IL-15 is produced by mouse PBMCs propagated in the presence of GM-CSF and IL-4, and its secretion and trans-presentation on IL-15R α by DCs promotes *in vitro* NK cell cytotoxicity and IFN- γ secretion (Lucas *et al.* 2007). Ferlazzo and colleagues (Ferlazzo *et al.* 2004) recently found that in humans, IL-15 production by monocyte-derived DCs or spleen DCs induces NK cell proliferation.

IFN- α and β also appear to be essential for the *in vitro* induction of NK cell cytotoxicity by various types of DCs (Walzer *et al.* 2005) (Figure 1-8).

As pointed out by one of the first studies on NK cells–DCs crosstalk (Fernandez *et al.* 1999), NK cell activation by DCs also requires direct cell-to-cell contact. This can be explained by the importance of the interaction of membrane-bound receptor–ligand pairs (Figure 1-7) and by the necessity for local delivery of cytokines at high concentration at the interface between DCs and NK cells.

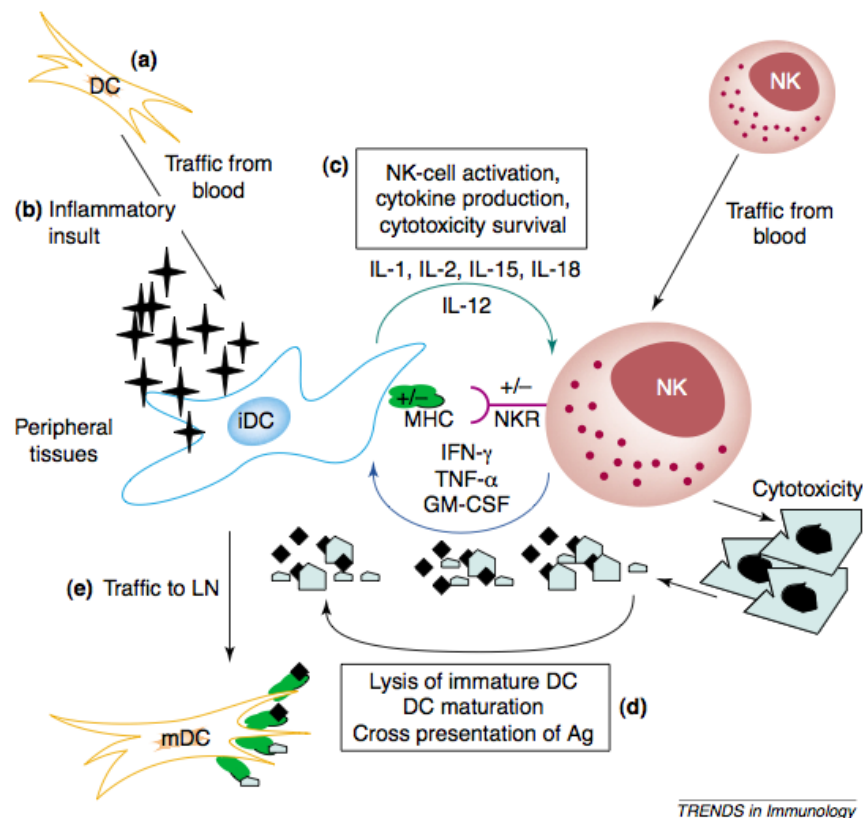


Figure 1-7 Interactions between NK cells and DCs in the periphery (Cooper *et al.* 2004).
Abbreviations: LN, lymph node; NKR: natural killer receptor; iDC: immature DC; mDC, mature DC

DC maturation induced by NK cells

NK cells are able to induce DC maturation either directly or in synergy with suboptimal levels of danger signals. This process appears to be dependent on TNF- α and IFN- γ secretion, and cell-to-cell contact is critical for optimal DC activation and subsequent induction of T cell responses. This process is particularly important in conditions where inflammation is poor (e.g, cancer), but where NK cell activation could occur through direct recognition of target cells (Walzer *et al.* 2005).

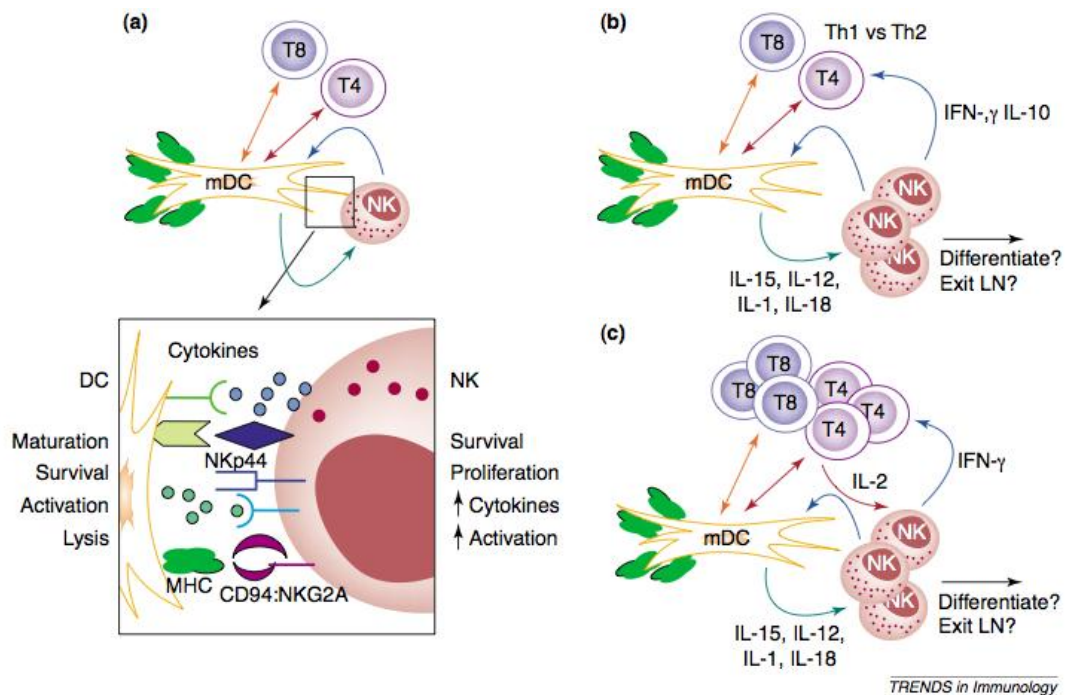
NK cells also exert a very important role in regulating DC homeostasis, and thereby the balance between tolerance and immunity, by killing immature DCs while sparing mature DCs. *In vitro* studies showed that signals delivered by NKp30, DNAM-1 and CD40 are critical for the lysis of immature DCs (Vivier and Biron 2002), whereas resistance to NK lysis is achieved by the up-regulation of MHC class I molecules during DC maturation, in particular HLA-E (Della Chiesa *et al.* 2003) (Figure 1-7).

Accumulating evidence suggests that the site of NK cell–DC interaction may also influence the outcome of the immune response. In lymph nodes, NK cells may contribute to DC activation and skew T cell differentiation, whereas NK cell–DC interactions in inflamed non-lymphoid tissues could promote NK effector functions or trigger DC maturation, as discussed in the following section (Walzer *et al.* 2005).

Role of NK–DC interactions in T and NK cell responses

In humans, CD56^{bright} NK cells are present in large numbers in lymph nodes where they have been shown to co-localise with DCs in the T cell areas (Bajenoff *et al.* 2006). This finding, together with the role of NK cells in DC maturation and activation, postulates a function for NK cells in the priming of T cell responses and in the polarisation of T cell responses through the secretion of high levels of cytokines in response to DC activation (Martin-Fontecha *et al.* 2004; Walzer *et al.* 2005) (see Figure 1-8). However, it is still not clear whether the cytokines produced by NK cells are directly affecting naïve T cells or are necessary for T cell priming by DCs, or both. Nevertheless, there is a suggestion that NK cell recruitment in lymph nodes might be important to license DCs to prime Th1 T cell responses by providing an early source of IFN- γ (Walzer *et al.* 2005).

Additionally, van den Broeke and colleagues (van den Broeke *et al.* 2003) have shown NK cell activation by DCs-activated CD4⁺ T cells. This phenomenon has also been confirmed in the context of MM, where immunomodulatory drugs (IMiDs) have been shown to activate NK cells through the release of IL-2 from CD4⁺ T cells (Hsu *et al.* 2011).



Role of NK–DC crosstalk in anti-tumour responses

NK cell activation by tumour cells has been shown to promote the initiation of cognate and protective T cell responses against tumours (Fernandez *et al.* 1999). The crosstalk between NK cells and DCs has, in fact, developed as a sophisticated switch to turn on innate and adaptive immune responses against most pathological conditions. In the case of a tumour that does not cause inflammation but does express ligands for activating NK cell receptors, NK cells would be the first cells to be activated and to subsequently provide help for DC maturation and the downstream activation of T lymphocytes (Degli-Esposti and Smyth 2005) (Fig. 1-9).

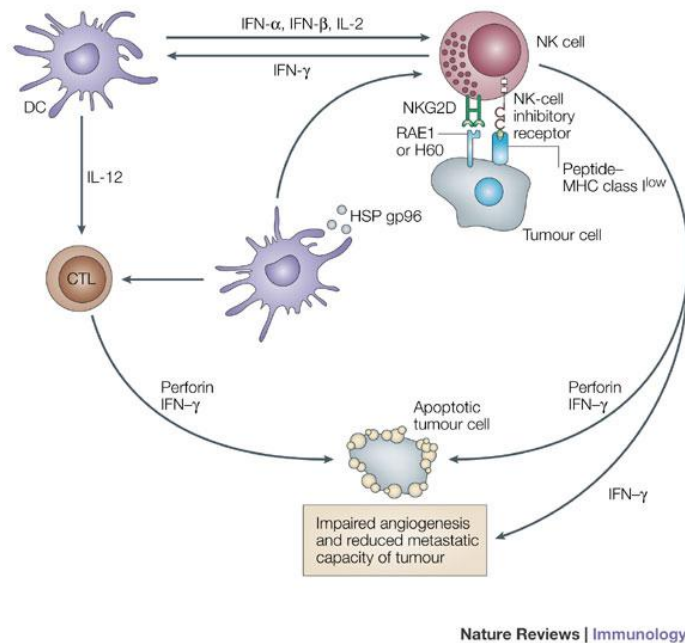


Figure 1-9 Role of DC cross-talk in the immune response against tumours (Degli-Esposti and Smyth 2005)

Interestingly, while freshly-isolated NK cells can recognise and kill tumour targets without the need for additional activation, NK cells can produce IFN-γ and induce the maturation of DC only when receiving an additional co-stimulatory signal. This second signal for the induction of NK cell “helper” activity can be provided by type-1 interferons, such as IFN-α and IFN-β, or by IL-2 produced by activated CD4⁺ T cells (Kalinski *et al.* 2005).

This more-stringent requirement for two-signals for the activation of NK cell “helper” functions, as opposed to their cytolytic “effector” function, may explain the poor effectiveness of immune responses against cancer. In fact, during early phases of tumour growth, NK cells are able to eliminate the transformed cells but, due to the lack of a second signal, they cannot perform their “helper role” and fail to support the development of type-1 tumour-specific immunity, resulting in the failure of tumour surveillance (Kalinski *et al.* 2005).

Therefore, the initiation of a proper immune response is dependent on the recognition of the pathological agent by either of the two ‘sensing’ partners and on their mutual crosstalk.

1.3 Immunodeficiencies in multiple myeloma (MM)

Several studies have highlighted the importance of the immune system in the disease progression of MM, particularly in the transformation from MGUS.

In fact, while displaying a genetic expression profile remarkably similar to MM, MGUS patients demonstrate a better ability to mount a vigorous T cell response when challenged with autologous plasma cells, compared to T cells from MM patients (Kyle and Rajkumar 2003). This phenomenon is likely to be the consequence, amongst many others, of the poorer expression of co-stimulatory molecules such as CD80 and HLA-DR (Perez-Andres *et al.* 2005), and activating molecules like NKG2D (Girlanda *et al.* 2005) by plasma cells from MM patients.

These observations provide strong evidences as to the importance of the host immune system in controlling disease progression, and have prompted the investigation the mechanisms underlying MM-induced immunodeficiency in order to identify potential strategies to enhance the autologous immune response to MM (Pratt *et al.* 2007). Results from these studies have helped to identify two principal mechanisms through which MM inhibits the immune system: a cytokine-mediated and a cellular mediated mechanism (Fig. 1-10).

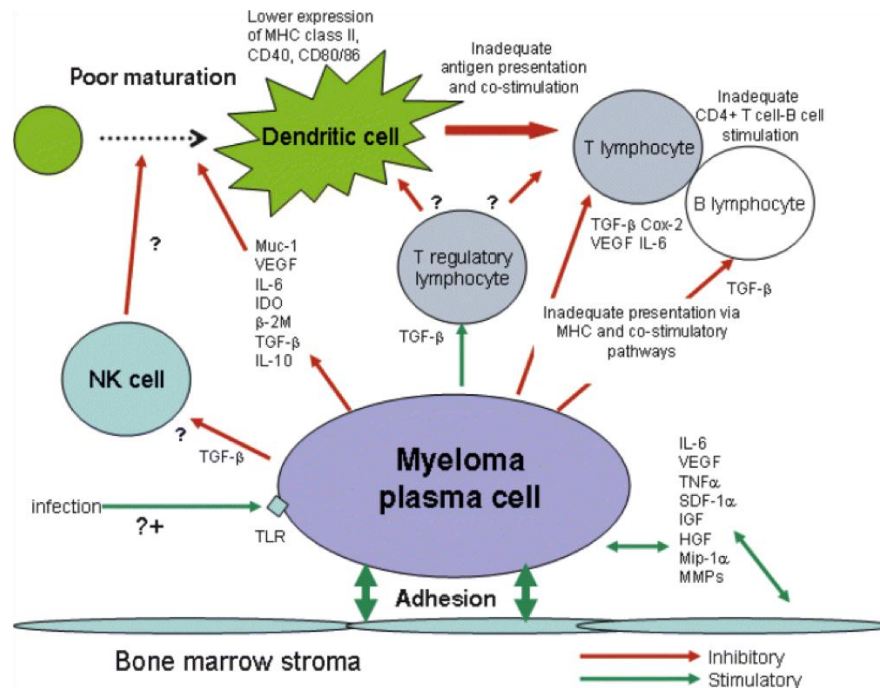


Figure 1-10 Interaction between MM cells and the immune system.

Abbreviations: VEGF, vascular endothelial growth factor; IL6, interleukin 6; IDO, indoleamine 2,3-dioxygenase; β 2M, β 2 microglobulin; TGF β , transforming growth factor β ; IL10, interleukin-10; TNF α , tumour necrosis factor α ; SDF-1 α , stromal-derived factor 1 α ; IGF, insulin growth factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteases; TLR, toll-like receptors (Pratt *et al.* 2007).

1.3.1 Cytokine-mediated immunosuppression

When considering the cytokine-mediated mechanisms involved in MM-induced immunosuppression, it is interesting to note that many of the molecules involved have a dual mechanism of action. In fact, they support tumour cell survival and drug resistance through the establishment of interactions with the bone marrow stroma, while promoting immune suppression. A number of immunologically active compounds have been identified, particularly within the bone marrow microenvironment.

IL-6 and L-10 are multifunctional cytokines, which play an important role in the biology of many tumours. In MM, they are involved in the inhibition of cancer cell apoptosis, stimulation of angiogenesis, and drug resistance (Pratt *et al.* 2007). In addition to these activities, they have also been linked to DC functional defects in MM patients (Brown *et al.* 2001), with IL-6 additionally promoting a humoral-mediated T helper cell type-2 response over a cellular mediated T helper cell type-1 response (Diehl and Rincon 2002).

Myeloma cells also produce high levels of Transforming Growth Factor beta

(TGF- β), which suppresses B and T cells through the inhibition of IL-2 autocrine pathways (Pratt *et al.* 2007), while stimulating the proliferation of T regulatory cells (Treg) (Braga *et al.* 2012).

The Vascular Endothelial Growth Factor (VEGF) has a fundamental role in promoting growth, survival and migration of myeloma plasma cells. Additionally, it was also shown to inhibit DC maturation and to elicit the production of IL-6 (Pratt *et al.* 2007).

The production of the glycoprotein Muc-1 has been shown to skew the differentiation of DCs into cells with a regulatory phenotype (Monti *et al.* 2004; Rughetti *et al.* 2005) whilst acting as a chemo-attractant for immature DCs (Carlos *et al.* 2005).

The high secretion of cyclooxygenase-2 (Cox-2) and prostanoids is considered a poor prognostic marker in MM. This is due to the fact that these molecules are responsible for tumour cell proliferation and survival, but also for negatively influencing anti-tumour immunity. This function is achieved through the induction of myeloid-derived suppressor cells, which inhibit T cell responses, NK cells and macrophage functions while expanding Treg numbers (Obermajer *et al.* 2012).

The activation of the immune response against MM is also suppressed through the release of matrix metalloproteinases (MMPs). These molecules, originally thought to be involved only in tumour invasion, have been also shown to have immunomodulatory properties as they prevent NK and T cell activation by shredding activating receptors from their cell surface and by inducing the release of immunomodulatory compounds (Gialeli *et al.* 2011).

1.3.2 Cellular-mediated immunosuppression

A second mechanism responsible for immune suppression is achieved through the expression of immunomodulatory receptors or ligands on the surface of MM and immune cells.

Fas Ligand (FasL) can normally be found on activated T cells, where its cross-link with Fas receptor induces apoptosis of the target cells. However, T and NK cells from MM patients have been shown to express significantly higher levels of Fas receptor, while MM cells present high levels of FasL and are therefore able to induce T cell and NK cell

apoptosis (Villunger *et al.* 1997; Poggi *et al.* 2005; Pratt *et al.* 2007).

In MM the presence of an imbalance of the Programmed Cell Death 1 (PD-1)/PD-L1 axis, which plays a key role in modulating the balance between tolerance and immune activation, has also been observed. Specifically, MM cells up-regulate the expression of PD-L1 in the presence of IFN- γ (Liu *et al.* 2007) while T (Rosenblatt *et al.* 2011) and NK cells (Benson *et al.* 2010) from MM patients present high levels of its ligand PD-1 and are therefore susceptible to PD-L1-induced cell apoptosis.

The expression of HLA-G on myeloma cells has been proposed to be relevant to tumour escape by directly conferring resistance to NK cell-mediated killing, and by affecting other immune effector cells such as T lymphocytes and DCs (Maki *et al.* 2008).

Additionally, MM cells have also developed a poor capacity to present antigens to the immune system. Their expression of HLA-DR and MHC class I (Pratt *et al.* 2007) as well as that of co-stimulatory molecules such as CD80 and CD40 (Yi *et al.* 1997) are often down-regulated.

1.3.3 Cellular immune defects in MM

The suppressive microenvironment created by MM leads to several cellular immune defects.

The cytokine milieu produced by tumour cells, including MM, blocks DC differentiation and attracts immature DCs. As a consequence, the majority of peripheral blood DCs from MM patients present an immature phenotype and, when stimulated, fails to up-regulate the expression of the co-stimulatory molecule CD80 (Brown *et al.* 2001).

This phenomenon also has an impact on T cells which, in MM patients, present a skewed CD4 to CD8 ratio (Pratt *et al.* 2007) and reduced cell numbers, possibly owing to the concurrent effects of IL-6, IL10, TGF- β , FAS-L and PD-L1 (Raitakari *et al.* 2003).

It has been shown that several types of tumours activate Treg in order to down-regulate the immune response (Joshua *et al.* 2008). However, although the MM cytokine microenvironment has the capacity to support the expansion of Treg and there is evidence demonstrating direct induction of Treg cells by both fresh myeloma cells and cell lines *in vitro* (Feyler *et al.* 2012; Feyler *et al.* 2013), there is still significant

disagreement in the literature concerning *in vivo* Treg numbers and function in myeloma patients (Braga *et al.* 2012). This discrepancy is probably due to differences in assay and purification techniques (Joshua *et al.* 2008) and, therefore, will require further investigations.

NK cells have an important role in the lysis of tumour cells, including MM plasma cells (Carbone *et al.* 2005; Harrison and Cook 2005). However, during the transition between MGUS and MM, malignant plasma cell sub-clones with low expression of immunostimulatory molecules, such as NKG2D, and increased levels of inhibitory receptors, such as HLA-G, MICA, PD-L1 and FASL, emerge (Raitakari *et al.* 2003; Carbone *et al.* 2005; Maki *et al.* 2008). These plasma cells have the potential to induce NK cell apoptosis and to cause the loss of inflammatory functions.

In view of this suppressive environment produced by MM cells and their microenvironment, several strategies to enhance autologous responses to MM have been examined (see chapter 1.4).

1.4 Immunotherapy for MM

Immunotherapy holds promise for treating several types of cancers, including haematological tumours as, by using different mechanisms of action, it has the potential to target the chemotherapy-resistant disease. These strategies are more likely to be effective in the setting of minimal residual disease following chemotherapy or haematopoietic stem cell transplantation (HSCT) as either consolidation or maintenance. The unique potential of immunotherapy for the treatment of MM is supported by the higher rate of molecular remission and lower rate of relapse and disease progression that are observed when comparing patients treated with allogeneic HSCT or donor lymphocyte infusion and those treated with autologous HSCT (Harrison and Cook 2005). Results reported by the European Group for Blood and Bone Marrow Transplantation, derived from 229 MM patients who received allogeneic HSCT with RIC regimens from 33 clinical centres (Crawley *et al.* 2005), suggest that the establishment of an allogeneic T cell response, demonstrated by the presence of chronic graft versus host disease (GVHD), is associated with better overall-survival and progression-free survival (Rutella and Locatelli 2012). Anti-tumour immunotherapy has therefore the potential to provide a powerful intervention to maintain long-lasting control of minimal residual disease or to even eradicate disseminated tumour cells.

Some immune therapeutic strategies have achieved clinical success and have been approved for clinical use for non-Hodgkin's lymphoma and prostate cancer (Zhang *et al.* 2012). Nevertheless, an immunotherapy protocol with unequivocal clinical benefit is yet to be established in MM (Zhang *et al.* 2012).

Despite the success of HSCT in other haematological malignancies, the use of allografting in the context of MM is limited by the high treatment-related morbidity and mortality (Harrison and Cook 2005). Several strategies have been tested to overcome this problem, such as the use of a T cell depleted graft followed by donor leukocyte infusion (DLI) (Alyea *et al.* 2001), the use of reduced-intensity conditioning regimens (Rosenblatt and Avigan 2008), and the administration of high dose autologous stem cell transplants in conjunction with high-dose melphalan therapy, followed by a

non-myeloablative reduced-intensity allogeneic transplant from HLA-identical sibling (Maloney *et al.* 2003).

Nevertheless, separating the graft versus myeloma effect (GVM) from the graft versus host disease remains a challenge. Orsini and colleagues demonstrated that GVHD and the GVM effects are mediated by distinct T cell populations that emerge at different times following DLI (Orsini *et al.* 2000). Using spectrotyping analysis and functional characterisation of the T cell receptor repertoire following donor leukocyte infusion, this group showed that one of the GVM clones was able to specifically recognise CD138⁺ MM cells but not CD138⁻ bone marrow cells in an HLA class-I-dependent manner. Therefore, on the basis of these results, future studies should aim to segregate the GVM effect from the GVHD effect through the identification and targeting of unique MM antigens by immune effector cells.

However, the establishment and growth of myeloma cells, despite the existence of T cells specific for tumour-associated antigens (TAA) in cancer patients, suggests that the presence of their targets is not sufficient to elicit a protective immune response. This might be due to a lack of tumour-reactive effector cells in the immune repertoire, or to the inability to expand and activate these cells effectively, due to the presence of a tumour-immune suppressive microenvironment. Following these considerations, the development of effective immunotherapies for myeloma should rely on the identification of appropriate tumour targets, the augmentation of antigen-presenting and effector cell functions, and the reversal of the tumour-mediated immunosuppressive state (Rosenblatt and Avigan 2008).

Within the last few years, a number of immunologically active compounds have been investigated *in vitro* and *in vivo* to test their ability to recover or increase the immune response against MM.

1.4.1 Humoral immunotherapy

1.4.1.1 Thalidomide and immunomodulatory drugs (IMiDs)

After its infamous banning in 1960s, thalidomide was rehabilitated for the treatment of multiple myeloma thanks to its anti-inflammatory, anti-angiogenic and immunomodulatory properties (Quach *et al.* 2010). Subsequently, analogues were synthesised with the aim of optimising thalidomide clinical effect while reducing toxicity. The two leading IMiDs compounds, lenalidomide (CC5-013, or IMiD3 or Revlimid) and pomalidomide (CC-4047 or IMiD1 or Actimid), are derived by adding an amino group to the fourth carbon of the phthaloyl ring of thalidomide (Quach *et al.* 2010).

Preclinical studies have unveiled multiple mechanisms of action for these compounds. Besides their ability to suppress plasma cell proliferation and disrupt MM-tumour microenvironment interaction, one of the most interesting and studied functions of IMiDs is the activation of the immune response (Quach *et al.* 2010). *In vitro* studies have, in fact, shown that IMiDs increase both the adaptive and innate immune responses by inducing the proliferation of partially activated T cells and the enhanced production of Th1 cytokines, such as IL-2 and IFN- γ , when compared to control conditions (Davies *et al.* 2001).

The molecular mechanism of T cells co-stimulation involves enhanced transcriptional activity of activated protein 1 (AP-1), a key driver for IL-2 production, which is then able to induce NK cell activation against MM cells (Davies *et al.* 2001). Additionally, LeBlanc and colleagues showed an increase in the tyrosine phosphorylation of CD28 on T cells (LeBlanc *et al.* 2004), which has the potential to lead to T cell activation.

This co-stimulatory effect of IMiDs has also been confirmed in an *in vivo* mouse model where IMiDs administered in the context of a tumour cell vaccination enhanced the protection against subsequent challenges with tumour cells (Dredge *et al.* 2002).

1.4.1.2 Monoclonal antibodies

Monoclonal antibodies are antibody molecules specific for a particular antigen that have been generated by a clonally expanded cell (Murphy 2011). The potential advantage of the use of monoclonal antibodies (mAbs) in MM therapy relies on their specificity for tumour-exclusive targets and their ability to recruit and activate cytotoxic effector mechanisms of the innate immune system (Danylesko *et al.* 2012). A variant of this strategy is the use of mAbs as targeted carriers of cytotoxic agents (Harrison and Cook 2005; Weiner *et al.* 2010).

Potentially valuable targets, which include molecules exclusively expressed on MM cells and involved in key tumour survival and migration (such as CD40, CD138, CD162, IL-6, VEGF) and immunomodulatory mechanisms (such as IL-6, VEGF, PD-1/PD-L1, CD200) have been evaluated over the past years. However, when employed as mono-therapy, these strategies have failed to produce impressive clinical responses. Nevertheless, results from preclinical *in vitro* and *in vivo* studies have recently suggested a potential synergistic effect when this approach is combined with traditional therapies (dexamethasone), immune modulators (thalidomide, lenalidomide), and other novel therapies such as bortezomib. Additionally, mAbs have shown the ability to overcome resistance to these conventional therapies (Danylesko *et al.* 2012).

An example of this phenomenon is provided by CT-011, a novel humanised IgG1 antibody against PD-1, which modulates immune response through disruption of the PD-1/PD-L1 axis. A phase 1 clinical trial of patients with advanced malignancies, including MM, showed that CT-011 administration as a single intravenous dose is safe, well tolerated, and is able to expand T cell subsets, with evidence of response in 33% of patients. Additionally, it increased migration of NK cells towards MM targets and enhanced the formation of immune synapses between patient-derived NK cells and PD-L1-bearing primary autologous MM cells (Berger *et al.* 2008). CT-011 also increases IFN- γ secretion and NK cytotoxicity against primary MM cells. Worthy of note, lenalidomide was shown to down-regulate PD-L1 expression on CD38⁺CD138⁺ primary

tumour cells, suggesting the employment of a combined immunotherapy with CT-001 and lenalidomide in MM (Rutella and Locatelli 2012).

These observations support the development of multi-agent therapies incorporating specific mAbs on the basis of clinical trial results and, possibly, on the identification of patient-specific MM disease factors (Danylesko *et al.* 2012).

1.4.2 Cellular immunotherapy

1.4.2.1 NK cells

NK cells play a major role in the development of the adaptive immune response thanks to their functional modulation of DCs (see paragraph 1.2.2.1.4). Resting NK cells, activated in the presence of IL-2 and IFN- α , are able to induce maturation of DCs in MM patients. These mDCs are characterised by enhanced production of IL-12p70 and are able to generate strong functional cytotoxic T lymphocytes (CTLs) against MM cells *in vitro* (Nguyen-Pham *et al.* 2012).

NK cells from MM patients have the potential to kill autologous MM cells and MM cell lines (Frohn *et al.* 2002). Adoptive transfer of activated NK cells to myeloma-bearing mice resulted in prolonged survival (Rosenblatt and Avigan 2008).

On this basis, a protocol to expand NK cells from MM patients *ex vivo*, in a good manufacturing practice compliant fashion, was optimised. After 20 days of culture, the number of NK cells was expanded and they were shown to kill autologous MM cells while cytotoxicity against normal cells was not observed (Alici *et al.* 2008).

Recently, it has also been shown that autologous NK cells from MM patients expanded *ex vivo* with IL-2 displayed significant cytotoxicity against primary autologous plasma cells. Furthermore, tumour-activated NK cells from MM patients were demonstrated to induce substantial lysis of myeloma cell lines, as well as autologous and allogeneic freshly isolated bone marrow malignant plasma cells (Katodritou *et al.* 2011).

Modulation of inhibitory and activating NK cell receptor ligands on tumour cells represents an additional promising therapeutic approach against MM. Proteasome inhibitors that target inhibitory KIR ligands on MM cells may contribute to the activation of cytolytic effector NK cells *in vivo* (Danylesko *et al.* 2012).

Depletion of Treg was also tested using cyclophosphamide in a mouse model. This strategy was successful in selectively depleting Treg while recovering NK cell and DC numbers and functions of (Sharabi and Ghera 2010).

1.4.2.2 T cells

Campbell and colleagues demonstrated that the *ex vivo* activation of T cells, using anti-CD3 monoclonal antibody in the presence of exogenous IL-15, was able to overcome MM-induced inhibition of T cells (Campbell *et al.* 2001a). However, these cells then became cytokine-dependent and failed to respond to antigens presented by DCs in the absence of IL-2 and IL-15.

Another problem for T cell-based therapies is the fact that the T cell repertoire in MM patients is severely skewed towards a type-2 T cell response. Autologous T cells activated with anti-CD3 and anti-CD28 beads and infused intra-venously after stem cell infusion succeeded in correcting the skewing of V β TCR repertoire and the lymphocyte count following bone marrow transplantation (Brown *et al.* 1997).

An additional interesting strategy could rely on the depletion of the suppressive Treg, which has been shown *in vitro* and *in vivo* to alter the Treg to T-effector cell ratio in favour of an anti-tumour response (Feyler *et al.* 2013).

1.4.2.3 DC-loaded with myeloma-associated antigens

DC-based vaccines have become an attractive tool for cancer immunotherapy and have been tested for the treatment of various malignancies such as melanoma, renal cell carcinoma, prostate cancer, and colorectal carcinoma (Nguyen-Pham *et al.* 2012). The potential of this strategy in the context of MM is supported by the identification of TAA-specific T cells in MM patients (see Table 1-2). These results suggest that T cells might be able to kill MM cells selectively, if a clonal population of T cells is activated and expanded effectively by a potent TAA.

The ideal TAA needs to be specific to the targeted tumour cell and, at the same time shared by a large proportion of patients. Additionally, it should be indispensable to tumour survival, in order to avoid escape through mutation or deletion, and be able to elicit a strong humoral and cellular immune response.

However, as the MM microenvironment interferes with DC functions, protocols for the generation of MM-specific DCs should involve the *ex vivo* culture of circulating blood precursors, which should then be educated and matured in the presence of tumour antigens and inflammatory cytokines prior to administration to patients.

1.4.2.3.1 Idiotypic (Id) proteins

In the context of MM, the antigen that appears to meet most of these requirements is the idiotype protein, which represents the variable segment of the monoclonal immunoglobulin generated by the plasma cell clone.

Early *in vitro* studies using this molecule showed the induction of both antibody and Id-specific CD4⁺ and CD8⁺ T cells, which were able to kill autologous primary myeloma cells *in vitro* (Wen *et al.* 2001b). However, despite these promising results and the induction of Id-specific CTLs *in vivo* in some patients (Danylesko *et al.* 2012; Nguyen-Pham *et al.* 2012), there has been no real evidence of clinical efficacy for this strategy (Harrison and Cook 2005).

Several clinical trials have subsequently attempted to improve the immunogenicity of this vaccination protocol through a combination with subcutaneous administration of cytokines. GM-CSF was tested alone or in combination with IL-12 (Rice and Hart 2002; Harrison and Cook 2005), and was able to elicit idiotype-specific T cell response and to significantly prolong time to disease progression. In one study, following autologous stem cell transplantation, MM patients were vaccinated with autologous idiotype protein conjugated with keyhole limpet haemocyanin (KLH), in conjunction with GM-CSF. Although humoral responses were observed and idiotype-specific delayed type hypersensitivity (DTH) skin tests were positive in 85% of the patients, residual disease was not eliminated (Coscia *et al.* 2004).

An alternative approach tested the immunomodulatory ability of T cells genetically-modified to express a TCR for the idiotype protein. Although these cells specifically killed primary MM cells *in vitro*, their *in vivo* efficacy was much more limited (Rosenblatt and Avigan 2008; Danylesko *et al.* 2012).

In view of the limited number of clinical responses observed and with the aim of overcoming the functional deficiencies of DCs in MM patients, the addition of *ex vivo* generated DCs to idiotypic-based vaccination strategies has been evaluated. The clinical application of this strategy was reported to be safe and to be able to induce both cellular and humoral immune responses and decrease in monoclonal protein (Reichardt *et al.* 1999).

However, the clinical outcome of these strategies is still overall disappointing, possibly because of the weak expression of idiotypic proteins, which, in the context of the immunosuppressive tumour microenvironment (Rosenblatt and Avigan 2008) fails to stimulate idiotypic-specific Th1 responses that are strong enough to control the growth of myeloma cells *in vivo* (Danylesko *et al.* 2012). Additionally, MM cells have been shown to induce tolerance to the idiotypic protein *in vivo* (Rosenblatt and Avigan 2008). These considerations prompted the study and identification of alternative antigens.

1.4.2.3.2 Tumour-associated antigens

Tumour-associated antigens (TAAs) have been identified and tested in many solid and haematological tumours. On the basis of the discovery of MM-specific T cells, many potential TAAs have been investigated, including polymorphic epithelial mucin (MUC1), human telomerase reverse transcriptase (hTERT), PRAME, HM1.24, SP17, WT-1, Dickkopf-1 and members of cancer germ-like family (MAGE and NY-ESO-1) (Nguyen-Pham *et al.* 2012).

This last family has been intensively studied in the context of myeloma and other tumours, given its limited expression in testis and placenta and, consequently its low risk of inducing autoimmunity secondary to immunotherapeutic strategies.

A summary of the principal TAAs that have been tested so far in the context of MM and the immune responses they were able to generate is reported in Table 1-2.

Table 1-2 Principal TAAs employed in MM-immunotherapy and stimulatory potential, adapted, expanded and integrated from (Zhang *et al.* 2012)

Antigen	Function	Expression in MM	Expression in normal tissue	Humoral response in MM	CD8+ T cell response in MM	Clinical trials in MM
MUC1	Multiple functions including surface barrier, and signal transduction	Fully glycosylated: 73%; Differentiation-dependent 59%; Cancer-associated glycoforms: 36%	Ubiquitous on the luminal surface of most simple epithelial cells	Natural antibodies to MUC1 have been detected in patients	Recognised by CTL in an MHC-unrestricted manner (Danylesko <i>et al.</i> 2012). MUC1 peptide-pulsed DCs or plasma cell RNA-loaded DCs (Nguyen-Pham <i>et al.</i> 2012) are able to generate CTLs specific for MUC1 expressing cell lines (Zhang <i>et al.</i> 2012)	Immunotherapy trial targeting MUC-1 peptide with GM-CSF (Danylesko <i>et al.</i> 2012; Zhang <i>et al.</i> 2012)
WT-1	Transcription factor	Frequent but low levels of expression	Placenta	N/A	Rise in WT-1-tetramer positive cells after 12 weeks of weekly intradermal injections of WT-1 peptide Myeloma cells can be recognised and lysed by WT-1-specific CTLs (Danylesko <i>et al.</i> 2012)	Decline in serum paraprotein and percentage of marrow plasma cells observed in one patients following weekly intradermal injections of WT-1 peptide vaccine (Rosenblatt and Avigan 2008; Zhang <i>et al.</i> 2012)
MAGE-C1	Spermatogenesis, cell cycle regulation	70-80%	Testis, placenta	Specific antibodies against MAGE-C1 were detected in 50% of AML patients with MAGE-C1 expressing MM cells. IgG antibodies detected in 10 out of 66 patients (9 out of 10 had undergone allogeneic HSCT) (Rutella and Locatelli 2012)	CD8 ⁺ T cell reactions against MAGE-C1 detected in MM patients. HLA-A2-restricted epitopes have been identified for MAGE-C1 and specific CD8 ⁺ T cells were able to recognise MAGE-C1 expressing MM cells (Zhang <i>et al.</i> 2012) CD4 ⁺ T cell reactions against MAGE reported but not in all MM patients.	Immunotherapy trial targeting MAGE-A3 peptide with GM-CSF (Danylesko <i>et al.</i> 2012; Zhang <i>et al.</i> 2012)
NY-ESO-1	Spermatogenesis, cell cycle regulation	Expressed in bone marrow of patients with MM but not in normal BM. Correlates with advanced disease	Testis, placenta	Spontaneous humoral response detected in patients with advanced disease. Antibody responses against NY-ESO-1 detected after allogeneic transplantation	Spontaneous CD8 ⁺ T cell response to NY-ESO-1 identified in patients with advanced disease (Nguyen-Pham <i>et al.</i> 2012). NY-ESO-1-specific CTLs from MM patients expanded by autologous DCs pulsed with NY-ESO-1-derived peptide, showed to kill primary MM cells, normal cells pulsed with a NY-ESO-1 peptide but not normal cells pulsed with an irrelevant peptide (Rosenblatt and Avigan 2008; Nguyen-Pham <i>et al.</i> 2012; Zhang <i>et al.</i> 2012)	Immunotherapy trial targeting NY-ESO-1 peptide with GM-CSF (Rosenblatt and Avigan 2008; Danylesko <i>et al.</i> 2012; Zhang <i>et al.</i> 2012)
Ropporin	Unknown	44%	Testis	Specific antibodies detected in the serum of ropporin positive patients (Zhang <i>et al.</i> 2012)	Specific CTLs were generated by incubation with autologous DCs loaded with ropporin and showed cytolytic effect against autologous MM cells (Zhang <i>et al.</i> 2012)	N/A
RHAMM	Receptor for hyaluronic acid mediated motility	100%	Testis, placenta, thymus	N/A	Rise in circulating cells binding to the RHAMM tetramer following vaccination with an immunogenic peptide derived from RHAMM (Rosenblatt and Avigan 2008);	Two phase I/II peptide vaccination trials including 7 MM patients with 3 showing clinical response (table) and 6 an increase in RHAMM-specific CD8 ⁺ T cells (Danylesko <i>et al.</i> 2012)
DKK1	Inhibitor of osteoblast differentiation	Almost all patients	Placenta, prostate and testis	Two anti-DKK1 neutralising antibodies have been tested as therapeutic agents in mice bearing human primary MM (Zhang <i>et al.</i> 2012)	DKK1-specific CTLs were detected in MM patients although at a low frequency and stimulation by autologous DCs loaded with DKK1 peptides generated specific T cells which were able to lyse DKK1-expressing primary cells in an HLA-A2 restricted manner (Zhang <i>et al.</i> 2012; (Nguyen-Pham <i>et al.</i> 2012)	N/A
HM1.24	Cell signalling	100%	Terminally differentiated T cells but its expression in normal tissue needs to be further investigated.	A humanised anti-HM1.24 monoclonal antibody has been developed and has exhibited anti-myeloma effect by inducing ADCC <i>in vivo</i> in a mouse model (Zhang <i>et al.</i> 2012)	HM1.24-specific CTLs can be induced <i>in vitro</i> in healthy volunteers and MM patients (Danylesko <i>et al.</i> 2012) by HM1.24-loaded DCs. Several HLA I-restricted epitopes within HM1.24 have been identified and proved to be of potent immunogenicity (Zhang <i>et al.</i> 2012)	N/A
hTERT	Human telomerase reverse transcriptase	85% (Vonderheide <i>et al.</i> 2001)	Limited expression in normal tissue (Carpenter and Vonderheide 2006)	N/A	Stimulation of CTLs by DCs loaded with hTERT-derived nonapeptide was capable of triggering anti-tumour CTL responses and kill hTERT ⁺ tumour cells (Rosenblatt and Avigan 2008; Nguyen-Pham <i>et al.</i> 2012)	Vaccine-primed T-cells early after transplant in conjunction with post-transplant boosters (pneumococcal-conjugated vaccine) is being evaluated
Sp17	Cell-to-cell adhesion and migration (Wen <i>et al.</i> 2001a)	26% (Lim <i>et al.</i> 2001)	Testis	N/A	Sp17-specific HLA class restricted CTLs were successfully generated by Dcs loaded with recombinant Sp17 protein. These CTLs were able to kill autologous tumour cells expressing Sp17 (Nguyen-Pham <i>et al.</i> 2012)	N/A

Nevertheless, none of these strategies resulted in a significant clinical response. These disappointing results may be explained by the fact that this approach shares the same limitations previously discussed in the context of idiotype vaccines (see 1.4.2.3.1). Several studies, which are employing bivalent or multivalent antigens and a wide range of immune adjuvants, such as NY-ESO-1 or MAGE-A3 peptides with GM-CSF or MUC1 peptide with GM-CSF, are still ongoing and their results should cast a new light on the potential and efficacy of this strategy (Zhang *et al.* 2012).

However, it is also worth noting that as most of MM immunogenic antigens are expressed on mature MM cells, this approach might spare tumour stem cells, which retain the capacity to self-renew, proliferate and lead to disease relapse (Harrison and Cook 2005).

1.4.2.4 Whole MM cells

1.4.2.4.1 DC loaded/fused with MM cells

Recent studies on tumour immunotherapy have revealed that the success of this strategy is limited by two main mechanisms: the selection of an edited tumour for loss of susceptibility to immune rejection, and a tumour edited immune system, already depleted of its most potent effector cells by chronic stimulation-mediated exhaustion and clonal deletion (Chan *et al.* 2006).

A possible alternative approach to improve the anti-tumour response and limit the risk of immunological escape may rely on the use of a broad panel of tumour antigens derived from whole tumour cells. This would allow the presentation of multiple epitopes on DCs, which would then have the potential to generate a polyclonal T cell response against known and unknown patient-specific TAAs. This strategy could also avoid the exhaustion and clonal deletion of TAA-specific T cells caused by the chronic immune stimulation provided by the tumour. In contrast, some of the weaker antigens, which may have failed to spontaneously induce an effective immunological response, could activate a reserve of unstimulated cells in the presence of the appropriate adjuvants and helper functions (Chan *et al.* 2006).

This strategy has been tested using different tumour preparations such as MM lysates, myeloma apoptotic bodies, DC transfected with myeloid-derived RNA and DC-myeloma hybrids (Nguyen-Pham *et al.* 2012).

DCs loaded with MM lysates were able to generate highly cytotoxic CTLs able to kill autologous tumour cells (Wen *et al.* 2002). Apoptotic bodies were interestingly shown to be more effective than cell lysates at inducing CTLs against autologous myeloma cells, possibly owing to the up-regulation of antigen presenting molecules (Nguyen-Pham *et al.* 2012).

DC/tumour fusions have the unique advantage to present a broad array of patients' specific and potentially unidentified antigens in the context of the antigen-presenting machinery of the DC fusion partner. After promising *in vitro* results the therapeutic potential of this strategy was evaluated in a clinical trial and shown to be well tolerated and to induce cellular and humoral immune responses. In particular, 17 out of 18 patients vaccinated with autologous DCs/tumour cell hybrids in combination with GM-CSF successfully responded to vaccination and 11 out of 15 evaluable patients showed an expansion of MM-reactive CD4⁺ and CD8⁺ cells (Rosenblatt and Avigan 2008).

The generation of a balanced CD4 and CD8-mediated response is crucial for the development of memory effector cells and the establishment of long-term anti-tumour immunity. Therefore, considering that cancer vaccines may increase the presence of regulatory T cells, one strategy to improve responses to vaccination involves the combination of active vaccination with adoptive transfer of CD3/CD28-expanded T cells. Furthermore, CD25⁺ regulatory T cells can also be depleted by specific monoclonal antibodies like denileukin difitox (Danylesko *et al.* 2012).

However, while it was possible to detect the induction of antigen-specific immune responses following virtually all these vaccination strategies, none of them was actually able to induce tumour regression. Recent studies have highlighted limitations of these strategies and, have consequently suggested new ideas for improving the efficacy of DC-based vaccination strategies (Table 1-3).

Table 1-3 Different anti-tumour vaccine strategies and related advantages and disadvantages. Adapted from (Harrison and Cook 2005)

Antigen	Advantages	Disadvantages
Tumour-associated proteins	Possibility to monitor the immune response to these antigen through the use of tetramers; ubiquitous antigens	Relies on the ability of a single antigen to be adequately processed and elicit a strong immune response.
Tumour lysates	Easy to prepare (freeze/thaw heat or sonication); produces a wide number of antigens available for processing by antigen presenting cells	Patient-specific; may expose antigen presenting cells to inhibitory molecules from the tumour; efficacy difficult to monitor.
Apoptotic (irradiation)/live tumour cells	All tumour antigens available	Patient-specific; how to convert tumour cells into efficient APC; efficacy difficult to monitor.
Tumour cells/DCs fusion	All tumour antigens available	Fusion process can be toxic to DCs; efficacy difficult to monitor.
Transfection of DCs with tumour DNA/RNA	Possibility to monitor the immune response to these antigen through the use of tetramers; ubiquitous antigens	Relies on high efficiency and low toxicity of the transfection process; relies on the ability of a single antigen to be adequately processed and elicit a strong immune response.

As MM patients present dysfunctional DCs due to the inhibitory effects of the MM microenvironment, several strategies have attempted to restore and enhance DC activation. The incorporation of Th1 polarising cytokines, such as IL-1 β , TNF- α , IL-6 and prostaglandin, in the vaccination protocol was successful in recovering and inducing the expression of co-stimulatory molecules and chemokine receptors. However, it also promoted Th2 polarisation, secretion of IL-10 and activation of Treg (Nguyen-Pham *et al.* 2012).

An alternative strategy relies on the inhibition of pathways involved in MM-induced impairment of DCs such as JAK2/STAT3 pathway, p38 activation, MEK/ERK pathway, MAK pathway and IL-6. This last strategy successfully rescued the abnormal phenotype and function of MM patient monocyte-derived DCs (Wang *et al.* 2006). More recently, this protocol has been further developed by Nguyen-Pham; by pre-treating MM cells with a combination of JAK2/STAT3 inhibitors and bortezomib they were, in fact, able to generate potent myeloma-specific CTLs (Nguyen-Pham *et al.* 2012).

An additional approach for the improvement of vaccination efficacy relies on the activation of DC migration to primary lymphoid organs, which can be potentiated by the synergistic effects of IFNs and TLR agonists. These molecules are in fact able to induce

the up-regulation of CD38, CD74 and CCR7 on DCs, which are required for DC mobilisation to lymph nodes (Nguyen-Pham *et al.* 2011).

Finally, following the promising results obtained in the context of idiotype vaccination, the use of lenalidomide was tested in conjunction with DC/myeloma hybrid cell vaccination. This resulted in the induction of Th1 immune responses and in the suppression of PD-1 expressing cells and Treg cells, supporting the potential of the combination of these strategies (Luptakova *et al.* 2013).

Combination approaches using DC-based vaccines to reduce tumour cells and immunomodulatory agents to overcome tumour microenvironment might therefore be helpful to improve the disease status.

Given the points discussed above, Nguyen-Pham and colleagues suggested that patients might benefit from vaccination strategies as illustrated in Figure 1-12 (Nguyen-Pham *et al.* 2012).

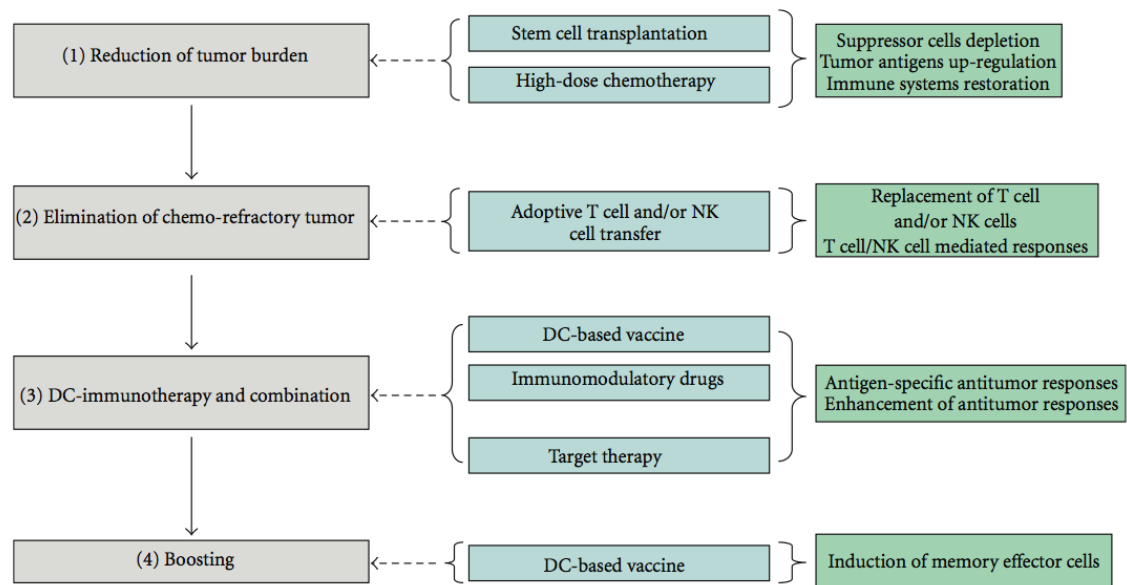


Figure 1-11 Suggested DC-based vaccine strategies for patients with MM (Nguyen-Pham *et al.* 2012).

However, since MM patients have generally insufficient numbers of myeloma cells in the bone marrow at the time of diagnosis or during the progression of the disease to provide with enough material for these therapeutic approaches and, considering the enormous cost and limitations of personalised therapies, allogeneic myeloma cell lines

are currently being investigated as a source of universal tumour antigens (Nguyen-Pham *et al.* 2012).

Nevertheless, despite the promising results and the encouraging advances reported, MM-DC fusion vaccination strategies still present disadvantages that need to be taken into consideration when evaluating their feasibility and optimising their clinical efficacy.

First of all, Harrison and Cook (Harrison and Cook 2005) highlighted the fact that the fusion process might be toxic to DCs. This issue, when considered in the context of the poor cell numbers and viability of MM patients' DCs after *in vitro* culture and the cost of the protocol, may represent a limit to the application of this strategy.

Another potential concern is the fact that the tumour fusion partner may inhibit the expression of co-stimulatory molecules on DCs or the maturation of the hybrid cell as an efficient antigen-presenting cell, as noted *in vivo* in patients with malignancy. This hypothesis is not supported by the results presented by Vasir and colleagues (Vasir *et al.* 2005) as they described that fusion of immature patient-derived DCs with autologous myeloma cells resulted in the marked up-regulation of expression of co-stimulatory and maturation markers. This was also associated with an increased expression of IL-12 and of the chemokine receptor CCR7, which directs migration towards the draining lymph nodes (Vasir *et al.* 2005). DC/myeloma fusions also stimulated T cell-mediated lysis of myeloma targets (Vasir *et al.* 2005). Further studies are needed to confirm these findings.

1.4.2.4.2 Whole cancer cell vaccines

A possible alternative to DC-based vaccines relies on the use of whole cancer tumour cells. However, this idea poses a great challenge: can tumours and, specifically, MM cells be converted to efficient antigen-presenting cells?

Vaccination with irradiated tumour cells has been studied in various animal models since the late 1970s. Hanna's group was the first to test irradiated hepatocellular carcinoma cells as a vaccine in a guinea pig model. This preparation was administered in conjunction with the general immune adjuvant *Bacillus Calmette-Guérin* (BCG) in an

attempt to boost the immunostimulatory ability of the tumour cells (Hanna and Peters 1978). This study was successful in generating protective immunity against a subsequent challenge with syngeneic non-irradiated tumour cells (Hanna and Peters 1978). These results also highlighted the importance of using intact tumour cells, suggesting either the direct involvement of the tumour cells themselves in antigen presentation or the importance of prolonged expression and release of TAA to allow the activation of immune effector cells at a site distant from the natural tumour microenvironment (de Gruijl *et al.* 2008).

Following these promising results, several clinical trials were conducted in the context of colorectal cancer and melanoma. The data collected showed a significant increase in overall and disease-free survival for vaccinated patients and delayed type hypersensitivity (DTH) reactions to autologous tumour cells, suggesting the induction of tumour-specific immunity (de Gruijl *et al.* 2008). Additionally, these observations strongly support the importance of administration of immunostimulating adjuvants in the effectiveness of booster vaccination. However, although BCG was proven to be a successful immune adjuvant, the observed ulcers at vaccination sites prompted the evaluation of alternative adjuvants for cancer vaccination.

The identification of hypomethylated CpG sequences derived from bacterial DNA as the immunoactivating component of BCG (Sandler *et al.* 2003) led to the design of a Phase II trial in which patients with advanced renal cell cancer were vaccinated with an autologous whole tumour cell vaccine mixed with a combination of CpG (type-B, PF-3512676), IFN α , and GM-CSF. This study showed a 20% clinical response rate in association with a strong anti-tumour DTH skin reactivity and demonstrated the equivalence of this adjuvant combination to BCG in terms of immune activation, without inducing ulcer formations (Haanen *et al.* 2006).

Following these encouraging results, scientists have been looking for alternative, targeted and controlled ways to achieve a cellular and humoral immune response following whole cancer cell vaccination.

It is important to highlight that the efficacy of the whole cancer cell vaccines

depends on two important events: the activation and maturation of antigen presenting cells, such as DCs, upon encounter with the vaccine, and the appropriate presentation of vaccine-derived TAAs to specific cytotoxic T cells. This consideration was the basis for the co-administration of immunostimulatory adjuvants along with tumour cells, at the site of vaccination. However, in order to maximise the efficacy of this strategy, whole cancer cells can be engineered to express and produce immunostimulatory molecules. The presence and release of high levels of immunomodulating cytokines in proximity with the tumour cells has, in fact, the potential of resulting in the effective stimulation of DCs, NK and cytotoxic T cells (Chan *et al.* 2006).

Based on these considerations and on the encouraging results obtained from preclinical studies, tumour cells transduced with GM-CSF (GVAX), have been tested clinically in a number of malignancies, including MM (Harrison and Cook 2005), and were shown to induce tumour-specific immunity and durable anti-tumour responses against melanoma, pancreatic and prostate cancer (de Gruijl *et al.* 2008).

While GVAX mono-therapy has shown moderate clinical efficacy, this may be improved by targeting checkpoints required for T cell activation. In 2000, Allison's group published a study where GM-CSF-secreting vaccine was administered in combination with a CTLA-4-blocking antibody. CTLA-4 regulates an inhibitory checkpoint for T cell activation and its blockage results in improved anti-tumour immunity and protection from tumour outgrowth in *in vivo* mouse models. These encouraging data have led to the initiation of a Phase I clinical study for prostate cancer (de Gruijl *et al.* 2008).

As T cell activation depends on the balance of activating and inhibitory signals, a possible alternative strategy to counteract tumour-mediated immune suppression and induce an effective cellular response might be to provide additional activating signals rather than neutralising the inhibitory ones. In order to increase their antigen-presenting ability, tumour cells may, in fact, be genetically modified to express the co-stimulatory molecule CD80. This receptor, which is often poorly expressed by tumour cells, has the fundamental role of providing the co-stimulatory signal necessary to induce T cell proliferation and activation following antigen presentation (Chan *et al.* 2006). Numerous

studies have demonstrated the effectiveness of increased expression of CD80 in anti-tumour immunity (reviewed in Table 1-4).

Table 1-4 Summary of studies investigating CD80-expressing cancer vaccines.

Tumour	Transgene	Experiments <i>in vitro/in vivo</i>	Results	Reference
ALL, AML, CLL	CD80 and GM-CSF	<i>In vitro</i>	T-cell proliferation in an autologous mixed lymphocyte reaction	(Stripecke <i>et al.</i> 2000)
AML	CD80	<i>In vitro</i>	Stimulate allogeneic CD4 and CD8 T cells	(Hirst <i>et al.</i> 1997)
	CD80	<i>In vitro</i>	Generation of leukaemia reactive CD4 ⁺ T-cell lines and clones.	(Mutis <i>et al.</i> 1998)
	CD80 and GM-CSF	<i>In vitro</i>	T cell expansion and activation	(Stripecke <i>et al.</i> 1998; Stripecke <i>et al.</i> 1999; Koya <i>et al.</i> 2002)
	CD80, GM-CSF and CD154	<i>In vivo</i>	T cell expansion and activation	(Vereecque <i>et al.</i> 2000)
	CD80 and IL-2 producing stromal cells	<i>In vivo</i>	<i>In vitro</i> anti-leukaemic response	(Hicks <i>et al.</i> 2003)
	CD80 and IL-2	<i>In vitro</i> and <i>in vivo</i>	T and NK cell expansion and activation	(Chan <i>et al.</i> 2005a; Chan <i>et al.</i> 2006; Ingram <i>et al.</i> 2009)
Breast cancer	CD80	<i>In vitro, in vivo</i> (Phase I/II)	Stimulation of CD8 ⁺ T cells, IFN- γ release	(Guckel <i>et al.</i> 1995)
	CD80 and IL12	<i>In vitro</i>	Propagation of T cells for adoptive transfers.	(Guckel <i>et al.</i> 1999)
	CD80 and HL with exogenous GM-CSF or BCG	<i>In vivo</i> (Phase I)	Safety and feasibility. Vaccine-induced immune responses in a minority of patients	(Dols <i>et al.</i> 2003)
Cervical carcinoma	CD80, IL7 and IL2	<i>In vitro</i>	Stimulation of cytotoxic T lymphocytes	(Kaufmann <i>et al.</i> 2000)
Melanoma	CD80, CD86, IL-12 (systemic)	<i>In vitro</i>	T cell proliferation	(Rudy <i>et al.</i> 1997)
	CD80 and CD86	<i>In vitro</i>	Lymphocytes proliferation with transcription of IL-10, IL-2 and IFN- γ . Blocking of IL-10 augments these effects.	(Dummer <i>et al.</i> 1998)
Mesothelioma	CD80 and GM-CSF	<i>In vivo</i>	T cell expansion and activation	(Mukherjee <i>et al.</i> 2001)
Metastatic renal cancer	IL-2, IFN- α	<i>In vivo</i>	Expansion and activation of naïve PBL <i>in vitro</i> . Is more effective in preventing tumour growth compared to systemic administration of IL-2.	(Belldgrun <i>et al.</i> 1993)
	IL-2	<i>In vitro</i>	Propagation of renal cancer cell-specific tumour infiltrating lymphocytes (TILs)	(Mulders <i>et al.</i> 1998)
	CD80 and systemic IL-2	<i>In vivo</i> (phase I)	Safety and acceptable toxicity; immunological and clinical response in some patients	(Antonia <i>et al.</i> 2002)
	CD80	<i>In vitro</i> (HD)	CD8 ⁺ T cell proliferation which were able to specifically lyse tumour cells	(Schendel <i>et al.</i> 2000)
	GM-CSF	<i>In vivo</i> (Phase I)	Positive DTH skin tests following autologous vaccination in all patients. Enhanced anti-tumour cellular and humoral responses.	(Tani <i>et al.</i> 2004)
	CD80 and IL-2 and IL-7	<i>In vitro</i>	Generation of CTL and proliferation of autologous effector memory T cells.	(Frankenberger <i>et al.</i> 2005)
	CD80 and IL-2	<i>In vivo</i> (Phase I)	Safety and feasibility. Vaccine-induced responses against multiple TAAs in the majority of study participants.	(Buchner <i>et al.</i> 2010)
	CD80, IL-7	<i>In vivo</i>	Vaccination feasible and safe; no indication of T cell activation	(Westermann <i>et al.</i> 2011)
Myeloma	CD80	<i>In vivo</i>	Rejection of CD80-expressing tumours	(Wendtner <i>et al.</i> 1997)
	CD80	<i>In vitro</i>	Allogeneic CD8 ⁺ T cell activation	(Tarte <i>et al.</i> 1999)
	CD80 and IL-12	<i>In vitro</i>	Enhanced lympho-proliferation	(Wen <i>et al.</i> 2001a)
	CD80 and CD154	<i>In vitro</i>	Enhanced proliferation and anti-tumour-specific response by CD8 ⁺ T cells	(Cignetti <i>et al.</i> 2005)
	CD80, p53 and GM-CSF	<i>In vitro</i>	Autologous CD8 ⁺ T cell expansion and activation	(Ren <i>et al.</i> 2006)
	CD80 and 4-1BB ligand	<i>In vitro</i>	Activation and expansion of MM-specific T cells	(Lu <i>et al.</i> 2007)
Prostate cancer	IL-2 and IFN- α	<i>In vivo</i> (Phase II/III)	Safe, well tolerated; prolongation of prostate specific antigen doubling time (PSA-DT)	(Brill <i>et al.</i> 2009)
Mouse sarcoma	CD80 and IL-2	<i>In vivo</i>	Prolonged survival	(Bubenik <i>et al.</i> 1997)

Dunussi-Joannopoulos and colleagues have shown that despite the presence of activated anti-tumour CD8⁺ T cells, CD80-based vaccines are able to eliminate only a relatively small tumour burden (Dunussi-Joannopoulos *et al.* 1998). Variables influencing this outcome may include the immunogenicity of the tumour, the tumour microenvironment surrounding the tumour and the type and amount of cytokine secreted. Therefore, in this respect, several groups have investigated whether transduction of tumour cells with additional molecules could enhance immunogenicity and vaccine efficacy. The results demonstrated that the combined expression of CD80 and stimulatory cytokines such as IL-2, IL-7 and GM-CSF, convert tumour cells into potent stimulators of both allogeneic and autologous T cells (reviewed in Table 1-4).

In the context of MM, following results by Wendtner (Wendtner *et al.* 1997) and Tarte (Tarte *et al.* 1999), which highlighted the potential of CD80 gene transfer in activating MM cell lysis by allogeneic T cells *in vitro*, a number of groups have addressed the potential of different cytokines to increase the CD80-mediated stimulation. Wen and colleagues showed enhanced allogeneic lympho-proliferation in response to co-culture of PBMCs with CD80/IL-12 modified U266 cells (Wen *et al.* 2001a). Cignetti's and Ren's groups, similarly, demonstrated that the lentiviral and retroviral transduction of primary MM cells was achievable and that the genetic transfer of CD80-CD40L or CD80-p53-GM-CSF turned MM cells into efficient antigen presenting cells, able to activate autologous cytotoxic CD8⁺ T cells (Cignetti *et al.* 2005; Ren *et al.* 2006). Finally, in 2007, it was shown that a T cell repertoire recognising myeloma tumour antigens persisted in the peripheral blood of MM patients, and that it could be activated and amplified by autologous tumour cells transduced with CD80 and 4-1BB ligand (Lu *et al.* 2007).

Due to the controversial role of CD80 in NK cell activation, (Geldhof *et al.* 1995; Galea-Lauri *et al.* 1999; Wilson *et al.* 1999; Luque *et al.* 2000; Terrazzano *et al.* 2002) none of these studies evaluated the effect of these strategies on NK cell expansion and activation. However Ingram and colleagues demonstrated NK cell expansion and activation following co-culture of PBMCs with IL-2/CD80 expressing acute myeloid leukaemia (AML) cells (Ingram *et al.* 2009).

Genetic modification of tumour cells

Gene therapy has achieved some important successes in treating severe inherited and acquired diseases (Naldini 2009). However, until recently, the application of this strategy has been limited by the safety concerns related to these procedures and the poor transduction efficiencies obtained using the available vectors.

Although many other agents such as calcium-phosphate, lipids and electric current are adequately used for gene transfer, viral vectors are endowed with a sophisticated machinery that facilitates efficient cell entry, transport and expression of their genome in the nucleus of target cells (Dropulic 2011).

Retroviruses were chosen for their ability to stably transduce cells by integrating their DNA into the cells genome (Dropulic 2011). A member of the Retroviridae family, lentiviral vectors, was selected and optimised on the basis of its efficiency in transducing both dividing and non-dividing cells, such as haematopoietic cells, its capacity for encoding large transgenes (up to 8kb) and its low potential for genotoxicity due to insertional mutagenesis (Hu *et al.* 2011).

Lentiviral vectors have been constructed from several types of lentiviruses, but the most commonly used is the human immuno-deficiency virus (HIV). These lentiviruses are composed of 2 copies of RNA, a nuclear capsid, a capsid, a membrane associated matrix, envelope proteins and transmembrane proteins and enzymes such as integrase, protease, reverse transcriptase, and accessory proteins (TAT, VIF, VPR, NEF and VPU). The HIV-based lentiviral vectors that are in use today have undergone improvements, which have mainly focused on increasing their safety and improving their tropism and transgene expression (Dropulic 2011).

Firstly, these vectors were proven to be safe due to the fact that most of the viral genes are removed from the viral genome. The necessary genes are provided in trans by transfecting the vector producing cells (helper cell) with plasmids encoding for viral proteins. This means that multiple recombination events are required in order to form a replication competent virus.

Additionally, the viral RNA carries a mutation in the 3' end. When the viral RNA is

transcribed into DNA inside the host cell, as part of the virus replication, it loses sequences at the 5' end and 3' end, which correspond to the promoter sequence and the Poly A signal. During the subsequent reverse transcription process that occurs to regenerate the viral RNA, the sequences on the 3' end are copied and placed on the 5' end through "jumps". As a consequence, the deletion present on the U3 segment of the original copy of the viral RNA is copied onto the 5' end of the final RNA, inactivating its promoter and, therefore, impairing the ability to form replicant competent lentiviruses.

HIV-1 based lentiviral vectors have also been optimised to be able to transduce a variety of dividing and non-dividing cells. This property is conferred by the presence of the VPR accessory viral protein and the use of Vesicular Stomatitis Virus G (VSV-G) glycoprotein, which enables the transduction of a wide range of cell types (Chan *et al.* 2005b).

Furthermore, several elements have been added to the transgene sequence in order to increase transgene expression. Incorporation of the HIV central polypurine tract (cppt) and termination sequence (cts) has been demonstrated to be important for the nuclear import pathway and for the transfer of viral DNA across the host cell nuclear membrane (Zennou *et al.* 2000; Follenzi *et al.* 2002). In addition, insertion of the woodchuck hepatitis virus posttranscriptional regulatory element in the 3' untranslated region of the lentiviral vector significantly increases the expression of the transgene as a result of improved intracellular messenger RNA stability (Zufferey *et al.* 1998). Finally, the incorporation of the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) along with lineage-specific U3 promoter sequences such as that of the spleen focus-forming virus, ensures the promotion of high levels of transgene expression (Barry *et al.* 2000; Yam *et al.* 2002).

Another issue that has been addressed is the development of strategies to efficiently concentrate lentiviral vectors. As large quantities of vectors might be needed to achieve the desirable transgene expression, the use of concentrated virus is important to avoid its dilution in the culture media, which can negatively affect the transduction efficiency, and to remove the inhibitory factors produced by helper cells (Chan *et al.*

2005b). VSV-G-pseudotyped vectors have been extensively used because of the remarkable stability of VSV-G envelope protein, which allows vector concentration by ultracentrifugation (Strang *et al.* 2004). However, due to VSV-G toxicity at high concentrations (Sakuma *et al.* 2010), alternative methods of vector pseudotyping and concentration have been developed in recent years.

An interesting one takes advantage of the fact that, when the virus exits from the producer cell (budding), proteins present on the membrane of the virus-producing cells are introduced on the surface of the lentiviral vector (Chan *et al.* 2005b; Nesbeth *et al.* 2006). On this basis, Darling and his group (Chan *et al.* 2005b) have been able to produce biotinilated viruses, which were then captured and concentrated using streptavidin magnetosphere paramagnetic particles (PMP). The large increases in titers observed when using this protocol suggested the promotion of additional vector-target cell interactions in addition to the removal of inhibitory factors (Chan *et al.* 2005b).

A final issue that has been investigated is the efficient transduction of target cells with more than one gene. A well-established strategy is the use of internal ribosome entry sites (IRES). However, the instability of vectors when more than one IRES is used, restricts the suitability of IRES vectors to express multiple genes (Gaken *et al.* 2000). To facilitate the efficient co-transduction of both CD80 and IL-2, Gaken and colleagues developed a “fusogene” approach. This strategy consists of the expression of multiple gene products as a fusion protein from a single cistron (Gaken *et al.* 2000). The fusion protein contains recognition sequences for the ubiquitous endoprotease furin, which is located primarily in the Golgi. Furin cleavage results in the generation of biologically active constituents from the fusion protein. Thus soluble IL-2 is released extra-cellularly whilst transmembrane CD80 remains anchored at the cell surface. This strategy also avoids the problems associated with transduction of target cells with two single gene vectors, or promoter interference arising when using two separate promoters (Gaken *et al.* 2000).

1.4.3 Considerations and future directions

Despite the incredible potential illustrated in this chapter, immunotherapy for MM has yet to prove its clinical efficacy. There are many reasons underlying the failure in translating extremely promising *in vitro* results into effective therapies.

First of all, *in vivo* studies have been conducted as very small clinical trials, thereby limiting the possibility of obtaining statistically significant results. Furthermore, most of these studies have focused on patients with refractory disease, while these immunotherapeutic approaches are likely to be more effective in patients with low tumour burden (Harrison and Cook 2005).

Some strategies have not been taken to the clinic because of poor immunostimulatory activity *in vitro*. While it is understandable to focus on the best immunoactivating strategies, especially considering the suppressive activity of the tumour microenvironment, Harrison and Cook (Harrison and Cook 2005) rightly observed that there is no proof that a cancer vaccine has to stimulate a large number of T cells in order to initiate tumour rejection. T cell responses to tumour antigens may be of a low level and negative results obtained with most *ex vivo* assays may not exclude the beneficial effect of tumour-specific T cells *in vivo* (Chan *et al.* 2006).

Nevertheless, given the complexity of myeloma cell-microenvironment interactions, combination therapies might be required to increase cytotoxicity and overcome drug resistance. As suggested by Harrison and Cook (Harrison and Cook 2005) a good strategy may rely on the combination of chemotherapy and IMiDs to lower the disease burden, DC/whole cancer cell-based vaccine to prime the immune system with pre-stimulation of T cells to correct tumour-induced anergy, followed up by low dose sub-cutaneous IL-2 and/or IMiDs, to maintain the activation of the immune system.

Such protocols should include close immunological monitoring of patients to evaluate and confirm the safety of this strategy and to examine the effect of each component, individually and in combination, on T and NK cell expansion and activation and on Treg activation.

1.4.4 Aims of the thesis

The studies reported in Chapter 1 outline the presence of abnormalities in the immune system of MM patients. However, although the phenotype and aberrations of T cells in MM patients have been extensively studied, little is still known about NK cells. In addition, the immunotherapeutic approaches intended to enhance immunogenicity have so far targeted T cells, with only modest efficacy *in vivo*, highlighting the need for a different approach.

On these bases, I wanted to test the hypotheses that MM cells have the ability to inhibit NK cells and that this inhibition can be abrogated via the stimulation with a genetically modified whole cancer cell vaccine. Specifically, my aim was to delineate and explain the mechanisms underlying NK cell abnormalities and to define whether this inhibition is contact or non-contact dependent and whether it is specifically directed at NK cells.

Secondly, I wanted to optimise a protocol to genetically modify MM cells to express CD80 and IL-2. These genetically-modified cells were then used, alone or in combination with immunomodulatory drugs (IMiDs), to stimulate healthy donor and MM patient PBMCs and tested for their ability to overcome MM-induced inhibition of NK cells.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Preparation of media and solutions

Cell lines were cultured, according to the supplier specifications, in complete medium obtained by adding 10 mL of a 100x stock solution of penicillin/streptomycin (10000 IU of penicillin and 10000 µg/mL streptomycin; Sigma, UK) and foetal bovine serum (FBS) (10% or 20% volume/volume; Life Technologies, UK) to either 1 L of Dulbecco's Modified Eagle Medium (DMEM) or RPMI1640 (Sigma, UK).

Recombinant human IL-2, IFN- γ and IL-6 were purchased from Peprotech (USA). The vials were centrifuged at 2000 x g for 30 seconds and the lyophilised cytokines were reconstituted to a concentration of 10 mg/mL using 100 mM Acetic Acid (Sigma, UK) and deionised water (Sigma, UK) respectively. Using the relationship IU/mg defined in the certificate of analysis, each cytokine solution was further diluted to 1×10^5 IU/mL with Dulbecco's Phosphate Buffer Saline (DPBS; Sigma, UK) supplemented with FBS (10% volume/volume), aliquoted in to single use aliquots and stored at -80 °C for up to 12 months.

PBMCs were cultured in a complete medium constituted of X-VIVO 15 without gentamycin or phenol red (Lonza, UK) supplemented with 10000 IU of penicillin and 10000 µg/mL streptomycin and IL-2 to a final concentration of 30 IU/mL.

Primary human MM CD138⁺ plasma cells were cultured in AIM-V medium (Life Technologies, UK) supplemented with 10000 IU of penicillin and 10000 µg/mL of streptomycin. IL-6 (100 ng/mL) was added every 3-4 days (Cignetti *et al.* 2005).

Freezing medium was prepared as a mixture of FBS and dimethyl sulfoxide (DMSO; 20% volume/volume; Sigma, UK).

For magnetic cell sorting, a separation buffer composed of DPBS, FBS (0.5% volume/volume) and 2 mM Ethylenediaminetetraacetic acid (EDTA; Sigma, UK) was used.

A staining buffer composed of DPBS supplemented with FBS (1% volume/volume) was used during antibody staining and washing procedures.

2.1.2 Cell lines

The human cell lines employed in this study were purchased from ATCC (American Type Culture Collection) and cultured according to the supplier's recommendations in humidified incubators at 37 °C, 5% CO₂. Cells were tested once every two weeks for mycoplasma contamination (as described in 2.1.2.5)

2.1.2.1 K562

The chronic myeloid leukaemia cell line K562 was established in 1970 from the pleural effusion of a 53 year-old woman with chronic myeloid leukemia (CML) in blast crisis (Koeffler and Golde 1980). The principal characteristics of this cell line are summarised in Table 2-1.

K562 is used as a highly sensitive target for *in vitro* NK cell cytotoxic assays as it is devoid of the inhibitory MHC class I molecules whilst overexpressing activating ligands for NK cells (e.g: MICA, MICB, ULBP-1, and ULBP-2) (Li *et al.* 2008; Muntasell *et al.* 2010).

Table 2-1 Characteristics of K562 cells

Cell line	Disease status	Age, sex, specimen	EBV status	Immunology	Cytogenetics	Protein/mRNA overexpression or secretion
K562	CML in blast crisis	53, F, peripheral blood	Negative	CD3 ⁻ , CD13 ⁺ , CD15 ⁺ , CD19 ⁻ , CD33 ⁺ , CD71 ⁺ , CD235a ⁺	human hypotriploid karyotype without sharp mode;,-X,-3,+7,-13,-18,+3mar, del(9)(p11/13)	MIC-A/B, ULBP2

Cells were cultured in complete RPMI1640 supplemented with FBS (10% volume/volume).

2.1.2.2 MM cell lines

The establishment of MM cell lines, which took place mainly in the late '60s and '70s, has allowed major advances in the understanding of myeloma. So far, 112 MM cell lines have been established (Drexler and Matsuo 2000). MM cell lines display unique biological features: morphology of plasma cell appearance, medium requirements (often including cytokines, feeder cells or a higher percentage of FBS), slow growth rate (about 54 hours), low maximum cell density, poor cloning ability, "mature B cell immune-phenotype" and specific chromosomal aberrations (mostly including 14q32) (Drexler and Matsuo 2000).

Features of the different MM cell lines used in this work are shown in Table 2-2.

Table 2-2 Characteristics of MM cell lines (Drexler and Matsuo 2000)

Morphology	Disease status	Age, sex, specimen	EBV status	Immunophenotype	Cytogenetics	Protein/mRNA overexpression or secretion
U266	Refractory/Termin al MM	53, Male, peripheral blood	Neg	CD3 ⁺ , CD5 ⁺ , CD10 ⁺ , CD13 ⁺ , CD19 ⁺ , CD20 ⁺ , CD34 ⁺ , CD37 ⁺ , CD38 ⁺ , cyCD79a ⁺ , CD138 ⁺ , HLA-DR ⁺	human complex hypodiploid karyotype with 6.5% polyploidy	IgE, IL-6, TNF- β
RPMI8226	MM, at diagnosis	61, Male, peripheral blood	Neg	CD3 ⁺ , CD5 ⁺ , CD10 ⁺ , CD13 ⁺ , CD14 ⁺ , CD19 ⁺ , CD20 ⁺ , CD21 ⁺ , CD34 ⁺ , CD38 ⁺ , CD80 ⁺ , CD138 ⁺ , HLA-DR ⁺	human flat-moded hypotriploid karyotype with 7.5% polyploidy; t (6;14)	IgG, TGF- β
KMS12-PE	MM, at relapse or refractory	64, Female, pleural effusion	Neg	CD3 ⁺ , CD4 ⁺ , CD13 ⁺ , CD14 ⁺ , CD15 ⁺ , CD19 ⁺ , CD20 ⁺ , CD34 ⁺ , CD38 ⁺ , CD138 ⁺ , HLA-DR ⁺	human hypodiploid karyotype with 8% polyploidy; t (11;14) (q13;q32);	Cyclin D1, MYC, amylase

MM cells were cultured in complete RPMI1640 medium supplemented with FBS (20% v/v). The identity of these cells was also confirmed by evaluation of known surface phenotypic markers (Bataille *et al.* 2006) and reported in Table 2-3.

Table 2-3 Phenotypic characteristics of MM cells

Cell line	HLA-ABC	CD28	CD138	HLA-DR	CD56	CD20	CD19	HLA-DR
U266	100%	100%	99%	51%	Not expressed	Not expressed	Not expressed	51%
RPMI-8826	100%	100%	96%	96%	100%	Not expressed	Not expressed	96%
KMS12-PE	100%	95%	82%	28.4%	Not expressed	Not expressed	Not expressed	28.4%

These results show that CD138 and CD28 may be used as selective markers for MM cells.

On the basis of the high levels of expression of CD56 by RPMI-8226 cells, which may represent an issue for the isolation of NK cells (defined as CD138⁻CD3⁻CD56⁺) from the co-culture with MM cells, the cell line RPMI-8226 was not used in co-culture experiments with PBMCs.

2.1.2.3 293T cells

The 293T cell line is a variant of the adherent human embryonic kidney cell line HEK293. The widespread use of this cell line is due to its high transfectability, which is essential since the production of third generation lentiviral vectors requires the co-transfection of three or four plasmids. Additionally, these cells were generated by transformation of the parental cell line with a fragment of the adenovirus genome, which results in the constitutive expression of the SV40T antigen. This allows the accumulation at high copy number of plasmids carrying the SV40 origin of replication, which results in very high expression levels of the proteins encoded by the plasmids (DuBridge *et al.* 1987; Louis *et al.* 1997).

Cells were cultured in DMEM medium supplemented with FBS (10% volume/volume).

2.1.2.3.1 BL-15 cells

These cells are HEK293T cells co-expressing the LNGFRBAP fusion protein, which is constituted by the biotin acceptor peptide (BAP) fused to the extracellular domain of the low-affinity nerve growth factor receptor, together with the bacterial biotin ligase (BirA) (Nesbeth *et al.* 2006). In the presence of biotin-supplemented medium (100 μ M; Sigma, UK), this allows the endogenous metabolic biotinylation of a lysine residue in the BAP domain of the fusion protein, which is then transported to the cell surface. This characteristic is exploited to generate biotin-labelled lentiviral particles that can then be easily purified from the cell culture medium. In fact, when the lentiviral particles bud from these cells, they retain part of the cell-biotinylated surface and can therefore be purified using streptavidin paramagnetic particles (PMPs; see 2.2.2.2.2). Cells were cultured in complete DMEM medium supplemented with FBS (10% volume/volume).

2.1.2.4 Mycoplasma test by polymerase chain reaction (PCR)

All cells lines were routinely tested every two weeks for mycoplasma contamination by using the EZ-PCR mycoplasma test kit (Biological Industries, Israel). This procedure is based on the amplification of genus and species-specific mycoplasmal 16S ribosomal RNA (rRNA) sequences, which are naturally present in high copy numbers and are independent of gene expression (van Kuppeveld *et al.* 1992). The primer set allows the detection of various mycoplasma species: *M. fermentans*, *M. hyorhinis*, *M. arginini*, *M. orale*, *M. salivarium*, *M. hominis*, *M. pulmonis*, *M. arthritidis*, *M. bovis*, *M. pneumoniae*, *M. pirum* and *M. capricolum*, as well as *Acholeplasma* and *Spiroplasma* species.

Culture supernatant (0.5 to 1 mL) was harvested from confluent cell cultures and centrifuged at 250 x g for 5 minutes to pellet cellular debris. The resulting supernatant was transferred to a sterile microcentrifuge tube (Eppendorf, UK) and centrifuged at 20000 x g for 10 minutes in a microcentrifuge (Ependorf, UK) to sediment mycoplasma. After carefully decanting the supernatant, the pellet was resuspended with 50 µL of the Buffer Solution provided with the kit and heated to 95 °C for 3 minutes in a heating thermo block (Bibby, UK). PCR reaction mixtures were then prepared in PCR tubes according to Table 2-4.

Table 2-4 Mycoplasma PCR reaction mix

Tube name	Volume (µL)
Molecular biology grade H ₂ O	35
Reaction Mix	10
Test Sample	5
Total volume	50

The efficiency of this protocol was tested by substituting the test sample with 1 µL of the provided positive template control or molecular biology grade H₂O (Sigma, UK) respectively as a positive control and negative controls.

PCR reactions were performed in DNA thermal cycler (G-Storm GS1, UK) set for the conditions detailed in Table 2-5.

Table 2-5 Mycoplasma PCR cycle

Temperature (°C)	Time (seconds)	Comments
94	30	
94	30	35 cycles
60	120	
72	60	
94	30	
60	120	
72	5	
4	Infinite	

The presence of any amplified fragment was detected by agarose gel electrophoresis.

2.1.2.5 Agarose gel preparation and electrophoresis

This technique is based on the separation of PCR amplified products in an agarose gel, which allows to determine the presence and size of these products by comparison with DNA ladders.

Agarose gel was generated by adding agarose powder (Sigma, UK) to 1x Tris Acetate-EDTA (TAE) buffer (Sigma, UK) at a 1 to 100 ratio (weight/volume). The agarose in the solution was subsequently melted in a microwave at maximum power and stirred until the solution became clear. After letting the gel cool for approximately 2 minutes, ethidium bromide (Sigma, UK) was added (8 μ L per 100 mL of solution) and the gel was poured into assembled gel trays with a casting comb. The gel was left to solidify for 30 minutes and the combs were carefully removed.

The gel and tray were placed into a gel rig and 1x TAE buffer was added until the entire gel was covered. Subsequently, the gel was loaded with a mixture containing 15 μ L of the PCR product and 3 μ L of 6x gel loading dye (New England Biolabs, USA). DNA ladder (5 μ L; O'Gene Ruler, New England Biolabs, USA) was loaded in the first and last well to help determining the sizes of the PCR amplified DNA fragments. The gel was then run for 1 hour at 120 volts.

The gel was gently removed from the rig and placed on a UV light box (UVP GelDoc-IT, UVP, Cambridge, UK). After turning the UV light box on, the gel was photographed using the Polaroid camera.

2.1.3 Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs)

2.1.3.1 Isolation of PBMCs

MM patients and age-matched healthy donors were included in the study following the local ethics committee approval and written informed consent in accordance with the Declaration of Helsinki and the specifications of the Human Tissue Authority prior to sample donation.

For healthy donors, peripheral blood was obtained in the form of anonymised leukocyte cones of apheresis collection, supplied by the United Kingdom Blood Transfusion Service, after institutional review board approval. For MM patients, both peripheral blood and bone marrow aspirates were taken by a qualified nurse or consultant using ethylenediaminetetraacetic acid (EDTA) blood collection tubes (Becton Dickinson, Oxford, UK).

PBMCs from peripheral blood and BMMCs from bone marrow aspirates were isolated by density gradient centrifugation. Samples were diluted in a 1:1 ratio with DPBS and mixed gently. In a 50 mL falcon tube, 30 ml of this mix were overlaid onto 15 ml of Histopaque density gradient solution (Sigma, UK) and centrifuged at 800 x g for 20 minutes at room temperature with no brake. The resulting interface layer, containing PBMCs, was then aspirated into a new sterile 50 ml tube (BD Biosciences, UK) using a pasteur pipette (Elkay, UK). Histopaque was removed by diluting the obtained PBMCs in a 1:5 ratio with DPBS and by centrifuging the resulting solution at 300 x g for 10 minutes at room temperature with maximum brake. Cell pellets were then washed two more times with DPBS, centrifuged at 250 x g for 10 minutes and resuspended in X-VIVO 15 complete medium. Viability and cell counts were determined by trypan blue exclusion method, as this dye is able to selectively traverse the membrane of dead cells. 20 µl of the cell suspension was stained with 60 µl of 0.4% Trypan blue (Sigma, UK). 10 µL of this mix was then loaded onto a haematocytometer, and a microscope (Leika Microsystem, Germany) with magnification x40 was used to count the viable cells. Cells in four of the large squares were counted and the average number of cells per large

square was used to determine the cell count per mL of cell culture according to the formula illustrated below.

$$\text{Cells/mL} = \text{average no. of cells in a large box} \times \text{chamber factor} \times \text{dilution factor}$$

2.1.3.2 Selection of cryopreserved MM patients PBMCs

Patient samples stored in the departmental tissue bank were selected on the basis of the availability of peripheral blood low-density cells stored in DMSO (n=77 products corresponding to n=32 patients). Of these samples, only those taken at presentation were considered for this study (corresponding to n=18 patients). This decision was made in order to avoid the possibility that the immunomodulatory therapeutic regimen received by the patient may contribute or interfere with the results of this study. A final exclusion criterion was the availability of at least 1×10^7 cells, as this was the minimum number of cells required for the planned assays. This left n=10 patients (corresponding to n=14 samples). However, 4 patients were subsequently excluded due to insufficient number of cells recovered after thawing or because of bacteria contamination of the samples.

Table 2-6 reports diagnosis and World Health Organisation (WHO) classification for the MM patients considered for this study, which were also confirmed by morphology, cytogenetics and molecular analysis at the Department of Haematological Medicine, King's College London.

Table 2-6 Characteristics of MM patients

Research Code	Sample type	Date sample taken	Age on the day of sample	Comments	Diagnosis and WHO subtype	Cytogenetics (FISH)	Comments
RC-11-0143	Peripheral blood	14/06/2011	67	Prior treatments: Merck 088 Trial-Guy's Hospital.	Multiple Myeloma IgG Lambda	Not applicable	Insufficient viable cells for cell separation or growth in culture.
RC-12-0709	Peripheral blood	10/10/12	40	Sample isolated in our facility from fresh peripheral blood	Multiple Myeloma IgG Kappa	Myeloma panel probes tested on CD138 ⁺ cells (PC): 1q21, 1p32.3, IGH@, t(4;14), t(11;14),t(14;16), TP53 and ploidy for chromosomes 5, 9 and 15, 5q deletion and ETV6.	Normal results.
RC-12-0713	Peripheral blood	15/10/12	85	Sample isolated in our facility from fresh peripheral blood	Multiple Myeloma IgG Lambda	Cyto FISH Result: Myeloma panel probes tested on CD138 ⁺ cells (PC): 1q21, 1p32.3 and TP53.	Normal results.
RC-12-0714	Peripheral blood	17/10/12	65	Sample isolated in our facility from fresh peripheral blood	Multiple Myeloma IgG Kappa	Myeloma panel probes tested on CD138 ⁺ cells (PC): 13q14,13q34, 1q21, 1p32.3, IGH@, t(4;14), t(11;14),t(14;16), TP53 and ploidy for chromosomes 5, 9, 15, and 12, 5q deletion and ETV6. ABNORMAL RESULT: Gain of one copy of CCND1 [24/76], loss of one copy of the 13q14 and 13q34 loci [34/100], IGH//MAF fusion present [100/100]. All other probes normal.	The molecular cytogenetic abnormalities seen are consistent with a clonal plasma cell disorder. The signature is compatible with high-risk disease associated with shortened OS.
RC-12-0715	Peripheral blood	17/10/12	81	Sample isolated in our facility from fresh peripheral blood	Multiple Myeloma IgG Kappa	Myeloma panel probes tested on CD138 ⁺ cells (PC): 1q21, 1p32.3, t(4;14), t(11;14),t(14;16) and TP53 . ABNORMAL RESULT: Loss of one copy of 1p32[88/100], gain of one copy of CCND1[82/100], gain of two to three copies of MAF[62/80]. All other myeloma probes normal.	The molecular cytogenetic abnormalities seen are consistent with a clonal plasma cell disorder.
RC-12-0781	Peripheral blood and bone marrow	06/12/12	71	Sample isolated in our facility from fresh peripheral blood and bone marrow	Multiple myeloma IgG Kappa	Myeloma panel probes tested on CD138 ⁺ cells (PC): 13q14,13q34, 1q21, 1p32.3, IGH@, t(4;14), t(11;14),t(14;16), TP53 and ploidy for chromosomes 5, 9, 15, and 12, 5q deletion and ETV6. ABNORMAL result, gain of one copy of chromosome 5 and loss of one copy of chromosome 15 [90/100], gain of one copy of the CCND1 locus [71/100]. All other probes normal.	The molecular cytogenetic abnormalities are consistent with a clonal plasma cell disorder. The signature is compatible with standard risk disease but should be considered in the context of associated host features.

Cells were thawed as described in 2.1.4.1 and resuspended in X-VIVO 15 complete medium for immediate cell culture in the presence of 30 IU/mL of IL-2.

2.1.4 Primary MM cells

Between 2006 and 2010, CD138⁺ cells from MM patients were stored in the departmental tissue bank as cell pellets and tryzol lysates only as there were not enough cells to produce viable products. After 2010 the protocol was dropped due to the reduced number of patient samples received. However, it was possible to receive 10 mL of bone marrow aspirate from one MM patient (RC-12-0781). MM plasma cells were isolated by density gradient centrifugation as described in 2.1.3.1 and, subsequently, CD138⁺ cells were purified from bone marrow mononuclear cells using magnetic microbeads (Miltenyi Biotech, UK), as explained in 2.4.1. Cells were then cryopreserved as described in 2.1.4.1.

2.1.4.1 Cryopreservation and thawing of PBMCs and CD138⁺ cells

Freezing medium (see 2.1.1) and Nalgene® “Mr Frosty” box (Nunc International, UK) filled with isopropanol (Sigma, UK) were cooled to 4 °C. PBMC and CD138⁺ cells were resuspended in X-VIVO 15 complete medium and diluted 1:1 with cold (4 °C) 2x freezing medium to give a concentration of $0.5-1 \times 10^7$ cells/mL. The cell suspension was immediately aliquoted into labeled cryovials (Greiner, UK). Cryovials were then placed in pre-cooled “Mr. Frosty” and stored at -80 °C for 24 hours. After 24 hours cryovials were moved into a freezer box and kept in vapour phase liquid nitrogen storage (-196 °C) until required.

Cells were thawed using an unstirred waterbath (Nickel-Electro, UK) set at 37 °C as rapidly as possible and slowly resuspended in 50 mL of DPBS, supplemented with Benzonase (Merk Millipore, Germany) to a final concentration of 50 IU/mL to prevent cell clumping. Cells were immediately centrifuged at 300 x g for 10 minutes. Cell pellets were resuspended with 1 mL of DPBS, the resulting cell suspension was passed through a 70 µm cell strainer (BD Biosciences, UK) to eliminate clumps and debris and washed once more with DPBS. Cell numbers and viability were determined using the trypan blue exclusion method described in section 2.1.3.1.

2.2 Lentiviral vectors

2.2.1 Construction of lentiviral vectors

All lentiviral vectors were designed and optimised by Dr. Chan, King's College London, who kindly provided the plasmids necessary to produce the virus.

Lentiviruses are composed of two copies of RNA, a system of capsids, membrane associated matrix, envelope proteins and transmembrane proteins (encoded by *Gag* gene) and enzymes such as integrase (ecoded by *Pol* gene), protease, reverse transcriptase and accessory proteins. Due to safety concerns regarding the infectious nature of HIV, recent lentiviral packaging systems have the viral components separated into 3 or 4 plasmids. In this study, lentivirus particles were produced by co-transfecting 293T cells with three plasmids, using the calcium phosphate co-precipitation method.

- I. The helper construct, p8.9, which encodes structural and enzymatic proteins essential for the minimal packaging of a lentiviral vector (GAG-POL-ENV-TAT).
- II. The envelope construct, MDG, expressing the surface glycoprotein of an unrelated virus (VSV-G) to replace HIV-1 ENV. VSV-G also expands the tropism of the vectors and allows concentration of the vector by ultracentrifugation.
- III. The transfer vectors (Figure 2.1), which contain: the packaging signal Ψ , which is required for efficient encapsidation, the Rev-responsive element RRE, which is necessary for reverse transcription and the central polypurine tract cPPT, which is required for nuclear transport and integration into the target cell genomic DNA. Additionally, to ensure biosafety, the cis-regulatory sequences in the 3' LTR are completely removed from the U3 region. This deletion is copied to 5' LTR after reverse transcription, resulting in transcriptional inactivation of both LTRs.

For this study, the following transfer vectors were used:

- 1) R'SINctwSVB71 (LV.CD80) encodes human CD80 (1074-bp)
- 2) HR'SINctwSVIL-2 (LV.IL-2) encodes human IL-2 (459-bp)
- 3) HR'SINctwSVIL-2B71 (LV.IL-2/CD80) encodes the human fusion IL-2/CD80 protein separated by a furin cleavage site (Arg.Gly.Arg.Arg). Endoplasmic Reticulum (ER) transport of chimeric IL-2/CD80 protein is mediated by the signalling peptide located at the N-terminus of the protein.

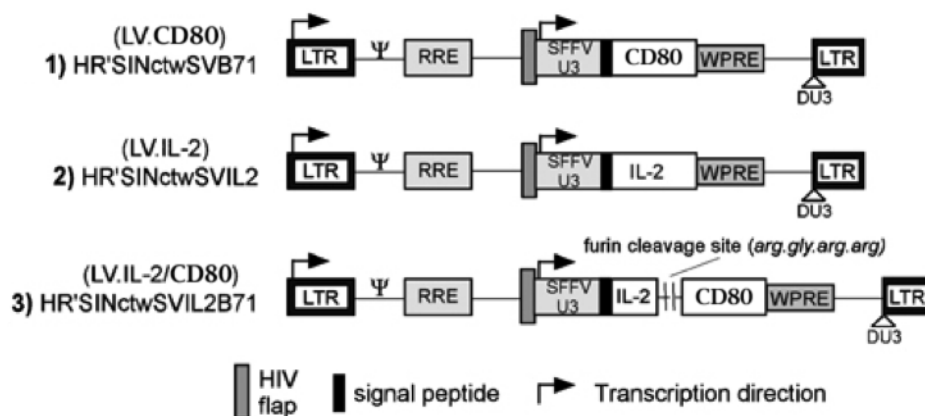


Figure 2-1 Schematic maps of the transfer vectors (Chan *et al.* 2005a)

The three plasmids also contain the *Bla* gene, which confers ampicillin/carbenicillin resistance and allows the plasmid to be expanded in Stbl3 *E.coli* cells in the presence of carbenicillin (see 2.2.2.1).

2.2.2 Lentivirus production

2.2.2.1 Plasmid expansion and purification

The plasmids described in 2.2.1, were expanded in Stbl3 *E. coli* cells. This strain is derived from the HB101 *E. coli* strain (Hanahan 1983) and was chosen as it is recommended for cloning unstable inserts such as lentiviral DNA.

2.2.2.1.1 *E. coli* transformation

The competent Stbl3 *E. coli* cells were purchased from Invitrogen (UK) and stored at -80 °C. On the day of the transformation, cells were thawed on ice and inoculated with the plasmid DNA (1-5 µL, corresponding to approximately 10-100 ng) or the pUC19 control plasmid, to check transformation efficiency, or water as a negative control. After a 30 minute incubation on ice, the mixture was heated for 45 seconds at 42 °C. This step

disrupts the membrane, allowing the DNA to enter the cell. The heated mixture was then placed back on ice to retain the plasmids inside the bacteria.

The transformed bacteria were subsequently resuspended in Super Optimal broth with Catabolite repression (S.O.C) medium (Invitrogen, UK) and shaken horizontally at 37 °C for 1hr at 225 rpm in a shaking incubator (Cole-Parmer, UK).

Each transformation mixture (25 and 100 µL) was then spread on pre-warmed Lysogen Broth (LB) agar plates (20g/L in dH₂O; Sigma, UK) supplemented with carbenicillin, an ampicillin analog (100 µg/mL; Sigma, UK) and incubated overnight at 37 °C.

2.2.2.1.2 Mini prep

In order to check that the carbenicillin resistant bacteria colonies were successfully transformed, at least two well-spaced single colonies were expanded in 5 mL of LB medium supplemented with carbenicillin (100 µg/mL) overnight at 37 °C, 225 rpm, in a shaking incubator.

Plasmid DNA was isolated by using the PureLink® Quick Plasmid Miniprep Kit (Life Technologies, UK) and tested for the presence of the desired insert by diagnostic restriction digestion (as described 2.2.2.1.3). 5 mL of the transformed and expanded cultures were lysed using an alkaline buffer provided with the kit (Life Technologies, UK). Subsequently, lysate was applied to a silica membrane column that selectively binds plasmid DNA while contaminants were removed using Wash Buffer. The plasmid DNA was then eluted in TE Buffer (Life Technologies, UK).

2.2.2.1.3 DNA purity and concentration evaluation

This procedure uses the absorption properties of DNA, which are detected by NanoDrop spectrophotometer (Thermo Scientific, UK), for quantification and assessment of DNA purity. After initialising the instrument using purified molecular biology grade water (Sigma, UK), the NanoDrop was blanked using 2 µL of the sample elution buffer. Subsequently, 2 µL of each sample were acquired and the absorbance, measured at

260 nm, was used to calculate the concentration of nucleic acids, based on the fact that at a concentration of 1 $\mu\text{g/ml}$ and a 1 cm path length dsDNA has a 260 nm absorption of 50 units. Table 2-7 shows an example of the spectrometric profile obtained for IL-2/CD80 plasmid DNA.

Table 2-7 Spectrometric profile of IL-2/CD80 plasmid DNA

Sample ID	Concentration (ng/ μL)	A260	A280	260/280	260/230
pLV.IL-2/CD80_1	1077	21.548	11.759	1.83	2.27
pLV.IL2/CD80_2	1099	21.973	11.950	1.84	2.28
pLV.IL-2/CD80_3	1117	12.235	12.235	1.83	2.23

Additionally, the A260/A280 ratio was used to determine protein ($\lambda_{\text{max}}=280\text{ nm}$) contamination. Pure DNA preparations have an A260/A280 ratio of greater than or equal to 1.8. The A260/A230 ratio was used as a secondary measure of nucleic acid purity as a strong absorbance around 230 nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. As a guideline, the A260/A230 ratio should be greater than 1.8, ideally close to 2.

2.2.2.1.4 Diagnostic restriction digestion

Two versions of the vector construct pLV.IL-2/CD80 were available in our department, which differ in the presence of the enhancer WPRE. Therefore, a diagnostic restriction digestion using appropriate endonucleases was carried out in order to check the identity of the expanded plasmid.

Using the DNA analysis tool Clone Manager (SciEd, USA), the restriction sites present in the plasmids and the predicted sizes of the resulting fragments were identified and calculated (Figure 2-2).

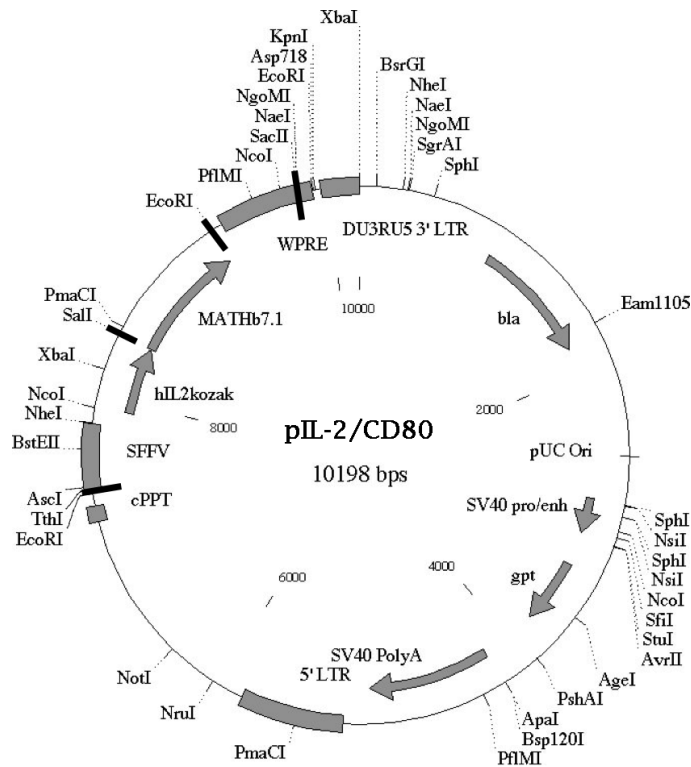


Figure 2-2 Restriction map of pLV.IL-2/CD80.

The enzymes *EcoRI* and *SalI* (New England Biolabs, MA, USA) were selected for their ability to excise part of the insert from the plasmid, which will have different sizes according to the presence or absence of WPRE (600bp).

The digestion reaction was carried in two steps, as the optimal buffer for the two enzymes differ for salt concentration, using a thermal cycler (GS1, G-storm UK).

Firstly, 200 ng of plasmid DNA was digested with the enzyme *EcoRI* using the reaction mix described in table 2-8. After 30 minutes at 37 °C, 0.5 µL of *SalI* were added to the mix and a higher salt concentration, which is required for the optimal activity of this enzyme, was generated by adding NaCl (1M; Sigma, UK). The reaction was run for another 30 minutes at 37 °C. Control samples were generated by digesting the plasmid DNA with only one enzyme or by adding molecular biology grade water.

Table 2-8 Diagnostic restriction digestion mix

	Volume (μL)			
	<i>EcoRI</i>	<i>SalI</i>	<i>EcoRI+SalI</i>	
			1 st step	2 nd step
DNA (200ng)	9	9	9	-
Buffer (NEB2; 10X)	2	2	2	1
BSA (1X)	2	2	2	-
NaCl 1M	-	1.5	-	1.5
Enzyme(s)	0.5	0.5	0.5	0.5
dH ₂ O	6.5	5	6.5	7
Total volume	20	20	30	

20 μL of the digested plasmid were then resolved by agarose gel electrophoresis as described in 2.1.2.6.

No further plasmid verification was performed, as all plasmids had already been tested by sequencing analysis (Chan *et al.* 2005a).

2.2.2.1.5 Maxi prep

Plasmids were further expanded by aliquoting 500 μL of one of the verified colonies (previously expanded, see 2.2.2.1.2) in 500 mL of LB medium overnight at 37 °C, 225 rpm in a shaking incubator. Cells were then pelleted by centrifugation at 4000 x g for 10 min and processed using the PureLink® HiPure Plasmid Maxiprep Kit (Life Technologies, UK).

Pellets were subjected to an alkaline lysis and lysates were then processed through a filter column, which includes a lysate filtration cartridge integrated into an ion-exchange column for single-step lysate clarification and plasmid DNA binding. Impurities such as RNA, proteins, and other contaminants were removed with a single wash, and plasmid DNA was eluted with a high-salt buffer (Life Technologies, UK). To precipitate and desalinate the DNA, isopropanol was added to the eluted DNA and the mixture was then applied to the HiPure precipitator using a syringe. After a subsequent washing and drying step, the plasmid DNA was eluted from the HiPure precipitator with TE buffer.

The obtained DNA was then tested for purity and concentration by absorbance spectroscopy, as described in 2.2.2.1.3.

2.2.2.2 Lentivirus production in helper cells

Lentivirus vectors (LV.CD80, LV.IL-2 and LV.IL2/CD80) were produced by co-transfection of helper cells (293T or BL15 cells) with three plasmids using the calcium phosphate precipitation principle. Details of the reaction mix are outlined in Table 2-9.

Table 2-9 Transfection reaction mix

Cell culture container	pMDG	p8.9	Transfer vector	H ₂ O	CaCl ₂	HeBS 2x
55 cm ² (10cm cell culture dishes)	3.5 µg	6.5 µg	10 µg	250 µL	250 µL	500 µL
435 cm ² (triple layer flask)	28 µg	52 µg	80 µg	1977.3 µL	1977.3 µL	3954.5 µL

Plasmid DNAs were mixed with water and CaCl₂ (Sigma, UK) and the mixture was slowly added drop-wise to a tube containing HeBS 2x (Sigma, UK) while vortexing the mixture continuously. The solution was then incubated at room temperature for 30 minutes to allow the formation of DNA precipitates and then added drop-wise to DMEM complete medium, which was used to replace the helper cells culture medium.

2.2.2.2.1 Lentivirus production and concentration using 293T cells

After 14-16 hours the culture medium was replaced with fresh complete DMEM to remove the excess DNA. Although the majority of the peak of accumulation of viral particles in the medium is at 24 hours post re-feeding, the data reported in Figure 2-3 suggested it is worth performing a second harvest. Therefore, supernatants were collected after 24 and stored at 4 °C and the medium substituted with fresh one. A second harvest was performed in the same way after another 24 hours (48 hours after the first re-feeding).

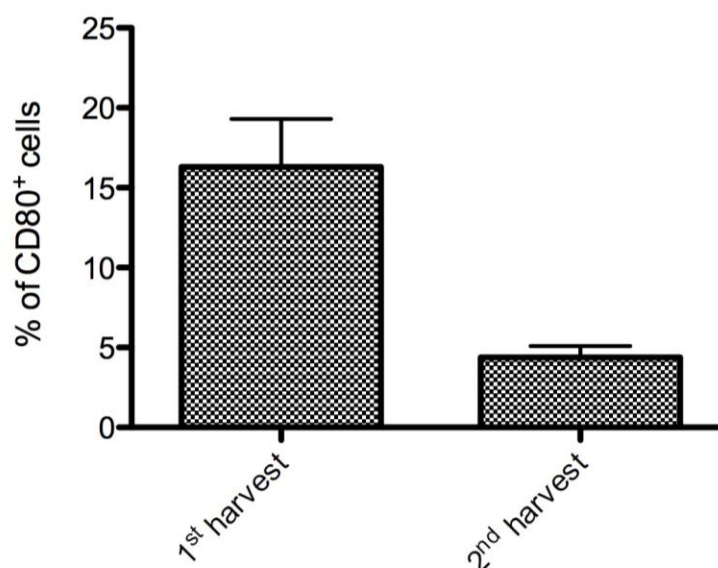


Figure 2-3 Detection of viral particles in first and second harvest following transfection of 293T cells.

293T cells were transfected with the appropriate plasmids as described in 2.2.2.2. 0.5 mL of supernatant, harvested after 24 (1st harvest) or 48 hours (2nd harvest) from the first re-feeding was used to infect K562 cells. After 24 hours, cells were washed twice and cultured for 72 hours prior to analysis of CD80 expression by flow cytometry. Bars represent the mean and standard deviation obtained from replicate wells.

Supernatants were then passed through a 0.45 μ m filter (Millipore, Germany), to avoid contamination by 293T cells, and concentrated in appropriately sized silicon tubes (Nalgene, Sigma, UK) by overnight ultracentrifugation at 8000 x g and 4 °C. Pellets were resuspended in AIM-V medium to achieve a total volume reduction of 100 fold, and frozen in 50 μ L aliquots at -80 °C.

2.2.2.2.2 Lentivirus production and concentration using BL-15 cells (Nesbeth *et al.* 2006)

24 hours after transfection (see 2.2.2.2), BL15 cells were washed three times with serum-free DMEM and cultured for 48 hours. Supernatants were harvested every 4 hours, stored at 4 °C and then passed through a 0.45 μ m filter. Calcium chloride (Sigma, UK) was added to the pooled supernatants and the mix was then incubated in an unstirred waterbath (Nickel-Electro, UK) at 37 °C for 30 minutes, resulting in a co-precipitated calcium phosphate-lentivirus preparation. This precipitate was then pelleted by centrifugation at 4000 x g for 5 minutes and resuspended in 900 μ L of modified solubilization buffer (100 mM EDTA, 50mM NaCl, 0.2% BSA, pH 6.5, all Sigma,

UK). Streptavidin paramagnetic particles (PMPs) (2.5×10^8 , Promega, UK) were added and the mix was incubated overnight at 4 °C under agitation using a roller mixer (Stuart Scientific, UK). After washing twice using a Dynal MPC-E (Invitrogen, UK), PMPs were resuspended in Hank's Balanced Salt Solution (HBSS; Sigma, UK) supplemented with FBS (0.1% volume/volume) to achieve a total volume reduction of 100 fold, and stored at 4 °C for further use.

2.2.2.3 Target cell transduction

2.2.2.3.1 Lentivirus titration

Target cells (K562, U937 and U266) were resuspended in complete RPMI1640 medium at 5×10^5 /ml and seeded in triplicate in 12-well culture plates. The infection enhancers diethylaminoethyl (DEAE)-dextran (10 µg/mL; Sigma, UK) or polybrene (4 µg/mL; Sigma, UK) was added to the medium and cells were cultured for 1 hour in humidified 37 °C, 5% CO₂ incubators. The polycation counters the repulsive electrostatic forces between the virus and target cells, thereby stabilising virus-cell interactions and thus virus adsorption to the cells (Platt *et al.* 2010). Serial dilutions of lentivirus particles or lentivirus-PMP complexes were added to the target cells. After 24 hours, cells were washed twice with fresh medium by centrifugation or by using a Dynal MPC-E (when PMPs-conjugated virus was used), re-fed with complete RPMI1640 medium and cultured for a further 48 hours. Cells were then washed and re-seeded at 1×10^6 /mL for a further 24 hours.

Flow cytometric analysis of cells for CD80 expression was performed (see 2.5.2) and supernatant removed for measurement of IL-2 concentration by Enzyme-Linked Immunosorbent Assay (ELISA), as described in 2.6.

Virus titer was calculated according to the following formula:

$$Titer \left(\frac{IU}{mL} \right) = No. of cells at the time of vector addition \times \frac{\% of CD80^+ cells}{100} \times dilution factor$$

2.2.2.3.2 Lentivirus infection of target cells

Cell lines U266 and RPMI8226 were seeded at a density of 5×10^5 cells/mL in complete RPMI1640 medium. Aliquots of the appropriate lentivirus were thawed and added at a

multiplicity of infection (MOI) of 20 in the presence of polybrene.

Cryopreserved CD138⁺ MM cells were thawed and cultured in complete AIM-V medium. Cells were then transduced with LV.IL-2/CD80 at an MOI of 20 in the presence or absence of the effector enhancer polybrene (4µg/mL).

After 24 hours, both infected primary CD138⁺ MM cells and cell lines were washed twice, re-fed with the appropriate complete medium and cultured for a further 48 hours. Cells were then washed and re-seeded at 1x10⁶ cells/mL for a further 24 hours, supernatants were removed for measurement of IL-2 concentration (as described in 2.6) and flow cytometric analysis of cells for CD80 expression was performed (see 2.5.2).

2.2.2.3.3 Target cells biotinylation and treatment with PEI

The polymeric transfection agent polyethylenimine (PEI; Sigma, UK) was combined with PMP-conjugated LV.IL-2/CD80 with the aim of maximising the lentivirus-target cell interaction (Chan *et al.* 2005b). Additionally, target cells were biotinylated to increase their ability to interact with PMP-lentiviral particles through the establishment of biotin (cells)-streptavidin (PMP) interactions.

U266 cells were resuspended in DPBS and incubated with a concentration of 3 mM of biotin for 30 minutes, washed twice and resuspended in fresh RPMI1640 complete medium. The presence of biotin linked to the cell surface was detected through staining with FITC-labelled avidin (Invitrogen, UK). U266 and biotinylated U266 cells were resuspended at a concentration of 5x10⁵ cells/mL in RPMI-1640 complete medium supplemented with polybrene (4µg/mL) and aliquoted in a 24 well plate (1mL/well). Each well then received an appropriate volume of PMP-conjugated LV.IL-2/CD80 in the presence or absence of PEI. After 24 hours, cells were washed twice using a Dynal MPC-E, re-fed with RPMI-1640 complete medium and cultured for a further 48 hours. Cells were then washed and re-seeded at 1x10⁶ cells/ml for a further 24 hours. Afterwards, supernatant were removed for measurement of IL-2 concentration and flow cytometric analysis for CD80 expression was performed.

2.3 Co-cultures

2.3.1 Co-cultures of PBMCs or NK cells with MM cell lines

Healthy donor PBMCs or isolated NK cells (see 2.4.1) were cultured for various durations in the presence of the MM cell lines KMS12PE, U266 and RPMI-8226 at a PBMCs to MM cells ratio of 1 to 1 and at an NK cell to MM cell ratio of 10 to 1. Typically, healthy donor PBMCs or isolated NK cells and MM cell lines were resuspended in their respective complete media at 2×10^6 cells/mL. 1 mL of each cell suspension was then aliquoted in a 12-well plate and IL-2 was added to a final concentration of 30 IU/mL. Cells were incubated in humidified 37 °C, 5% CO₂ incubators for 24 hours unless otherwise specified. CD20⁺ B cells isolated from HD PBMCs (as described in 2.4.1) or complete RPMI-1640 medium were used as negative control wells. In one experiment, contact-dependent interactions between PBMCs and U266 cells or CD20⁺ B cells were prevented through the use of transwell with 0.4 µm polycarbonate membrane insert (Corning, USA).

In order to prove the potential relevance of these *in vitro* results, MM patient PBMCs (2×10^6 at 2×10^6 cells/mL in complete X-VIVO 15 medium) were cultured for 24 hours in 1 mL of complete RPMI-1640 medium.

2.3.2 Co-culture of PBMCs with unmodified or genetically-modified MM cells

Unmodified or genetically modified U266 cells, or primary unmodified or LV-IL-2/CD80-modified primary CD138⁺ MM cells were washed twice in DPBS, resuspended in complete medium, γ-irradiated at 30 gray (Gy) and used as stimulator cells in co-culture experiments with healthy donor and MM patient PBMCs at a PBMCs to MM cell ratio of 2 to 1. Typically, PBMCs and MM cells were resuspended in the appropriate complete medium at 2×10^6 cells/mL and at 1×10^6 cells/mL, respectively. The cell suspension was then aliquoted in a 6-well plate and culture media was supplemented with 30 IU/mL of IL-2.

Cells were incubated in humidified 37 °C, 5% CO₂ incubators for 7 days unless otherwise specified. X-VIVO 15 complete medium was added in the negative control wells (PBMCs alone).

2.3.3 Culture of PBMCs in the presence of immunomodulatory drugs (IMiDs)

The immunomodulatory drugs pomalidomide (Actimid or CC-4047) and lenalidomide (Velcade or CC- 5013) were purchased from Sigma (UK), aliquoted as a powder in 3 mL polypropylene tubes (Eppendorf, UK) and weighted under sterile conditions. The aliquoted drug was stored at 4 °C and, when needed, resuspended in the appropriate amount of DMSO to reach a final concentration of 100 mM. Freshly isolated and thawed HD PBMCs were washed twice in DPBS and resuspended in complete X-VIVO 15 medium. The appropriate volume of drug resuspension or DMSO was then added to the culture medium and resuspended well by pipetting up and down three times. Cells were incubated in humidified 37 °C, 5% CO₂ incubators for the desired time.

2.4 Cell sorting

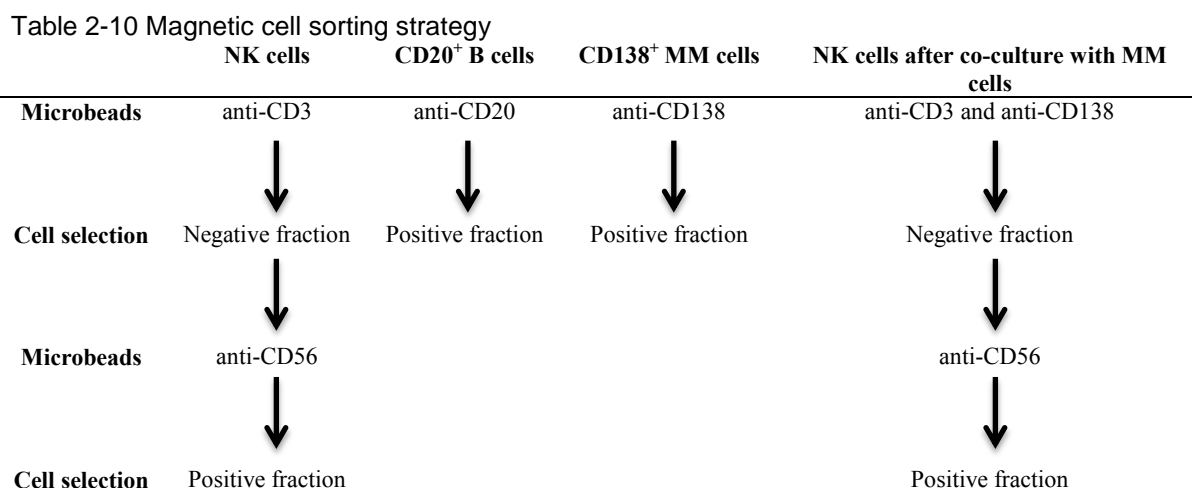
2.4.1 Magnetic cell sorting

Cell isolation was achieved by using a magnetic labelling system (Miltenyi Biotech, UK). This method is based on the use of biotin-conjugated antibodies to specifically label cells of interest. The labelled cells can then be depleted or enriched from a heterogeneous cell population by retaining them within a column in the magnetic field of a separator. Unlabelled cells run through the column while labelled cells are held into suspension within the column and can be subsequently eluted by removing the column from the magnetic field.

After determining cell numbers and calculating the quantity of reagents needed, cells were washed once with DPBS, resuspended in 5 mL of DPBS and passed through a pre-moistened 70 μm nylon cell strainer (BD Biosciences, UK) to remove cell clumps that may clog the column. Cells were centrifuged at 300 x g for 10 minutes and, after the supernatant was completely aspirated, resuspended in 80 μL of staining buffer (see 2.1.1) per 10^7 total cells. 20 μL of microbeads per 10^7 total cells were added and cells were incubated for 15 minutes at 4 °C. Cells were then washed using 10 mL of staining buffer and up to 10^8 cells were resuspended in 500 μL of staining buffer.

After choosing an appropriate MACS Column and MACS Separator (all from Miltenyi Biotech, UK), the columns were placed in the magnetic field of the MACS separator and rinsed with staining buffer. The cell suspension was then applied onto the column. After two washes the total flow through, representing the unlabeled cells, was collected and stored for further processing or use. The column was removed from the separator and placed on a suitable collection tube. The labelled fraction was immediately flushed out by firmly applying the plunger supplied with the column.

Table 2-10 describes the strategies used to isolate NK cells and CD20⁺ B cells from HD and MM patient PBMCs, CD138⁺ cells from MM patient BMMCs and NK cells following co-culture with MM cells.



Purity of the product and efficiency of the procedure was then evaluated by Flow Cytometry as described in 2.5.2 (Figure 2-4, 2-5 and 2-6).

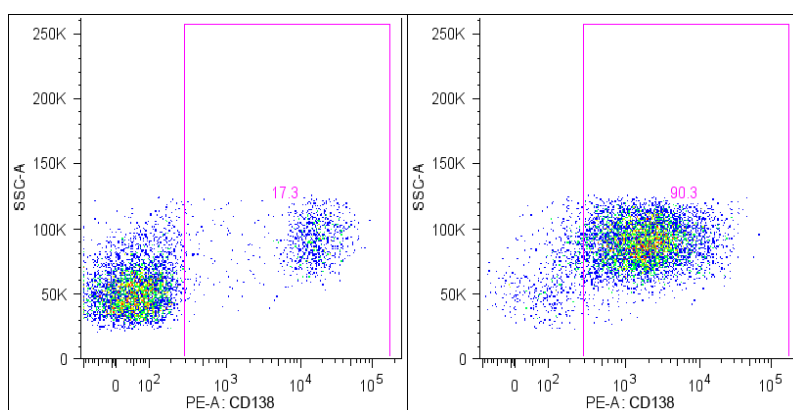


Figure 2-4 Purity of CD138⁺ cells before and after magnetic cell sorting. Flow cytometry plots showing CD138⁺ cells in the PBMCs before (left) and after (right) CD138⁺ cell selection using Miltenyi microbeads.

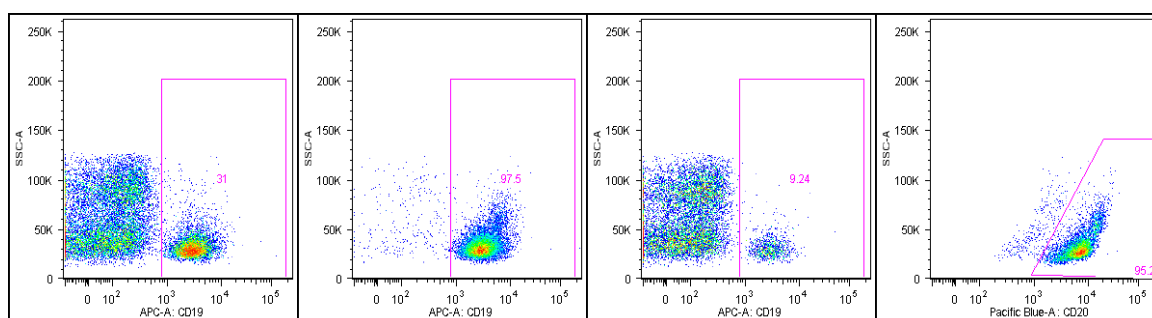


Figure 2-5 Purity of CD20⁺ B cells before and after magnetic cell sorting. Flow cytometry plots showing CD19⁺ cells (first, second and third panel) in the PBMCs before (first panel) and after CD20 B-cell isolation (second panel: positive fraction; third panel: negative fraction) using Miltenyi microbeads. The positive fraction was also tested for CD20 expression (fourth panel).

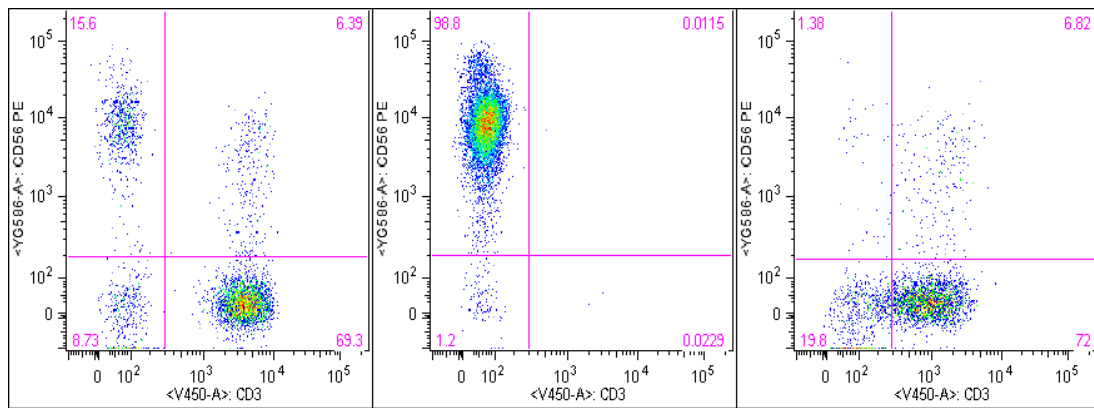


Figure 2-6 Purity of CD3⁺CD56⁺ NK cells before and after magnetic cell sorting. Flow cytometry plots showing NK cells (defined as CD3⁺CD56⁺) in the PBMCs before (first panel) and after CD3 depletion and CD56 enrichment (second panel: positive fraction; third panel: negative fraction) using Miltenyi microbeads.

2.4.2 Fluorescence activated cell sorting (FACS)

LV.IL-2/CD80 and LV.CD80 U266 cells were washed twice in DPBS. 5×10^5 cells were aliquoted in two polypropylene tubes (BD Biosciences, UK), one of which served as unstained control while the second received isotype control antibody (BD Biosciences, UK). The rest of the cells were stained with anti-CD80PE monoclonal antibody (BD Biosciences, UK). After 30 minutes incubation in the dark at room temperature, cells were washed twice with separation buffer and resuspended at $10\text{--}15 \times 10^6$ cells/mL. As it is fundamental that cells are in a single-cell suspension, cell clumps were removed right before the sorting by passing cells through a 70 μm cell strainer (BD Biosciences, UK). Cells were resuspended in a buffer containing cation-free DPBS with 1 mM EDTA, 25 mM HEPES (Sigma, UK) at pH 7, supplemented with FBS (1% volume/volume) and penicillin streptomycin. Cells were kept on ice to help maintaining a monodispersed suspension.

Collection tubes were prepared by aliquoting complete medium supplemented with FBS (50% volume/volume). FACS was performed using BD FACSariaTM III (BD Biosciences, UK).

Firstly, unstained and negative control samples were run to identify the positive and negative cell populations. The sample was subsequently run and the positive and negative populations were collected in the appropriate tubes. As part of the quality

control, both tubes were re-acquired to check the efficiency of the procedure and the purity of the sorted population.

2.5 Flow cytometry

Flow cytometry is a technique based on the use of multiple beams of laser light characterised by a specific wavelengths, to detect physical information about a cell suspension in the form of a hydrodynamically focused stream of liquid. A number of detectors are positioned at the point where the stream passes through the light beams: one in line with the light beams (Forward Scatter or FSC) and several perpendicular to them (Side Scatter or SSC) and one or more fluorescence detectors, which can detect any light emitted by fluorescent molecules excited by the light source. By analysing fluctuations in brightness at each detector it is then possible to derive information about the physical and chemical structure of each individual particle passing the light beams. FSC correlates with the cell volume, while SSC depends on the inner complexity of the particles (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness) as the light is scattered off the internal components of the cell.

Cells were acquired using the BD Canto II flow cytometer (BD Biosciences, UK) and data analysed using FlowJo Software (Treestar, USA).

2.5.1 Quality control of flow cytometric assays (Mahnke and Roederer 2007)

Cytometer Setup and Tracking beads (BD Biosciences, UK) were run once a week to control the performance levels of the cytometer. These beads consist of equal concentrations of 3 μm bright, 3 μm mid and 2 μm dim polystyrene beads in PBS with FBS in a stream-tip dropper vial. They were originally used to define the cytometer baseline, in particular to adjust the cytometer settings for maximising population resolution in each detector. Once baseline values were defined, 1 drop of beads diluted with 150 μL of DPBS was used weekly and after every machine servicing to evaluate cytometer performance.

Prior to each acquisition, compensation beads (BD Biosciences, UK) were run with the purpose of establishing compensation corrections for spectral overlaps for any combination of fluorochrome-labeled antibodies. BD™ CompBead particles are polystyrene microparticles coupled to an antibody specific for the kappa light chain of

mouse immunoglobulin. Each CompBead set also includes a negative control with no binding capacity, which consists of particles labeled with BSA or FBS. When mixed with any kappa-bearing fluorochrome-conjugated antibody, the beads can provide distinct positive and negative (background fluorescence) stained populations that can be used to set compensation levels. This method has the advantage to provide a bright and distinct population of positive cells, thereby overcoming the problems associated with the dim or rare expression of a marker on normal cells and avoiding the use of valuable cell samples.

A separate FACS tube (BD Biosciences, UK) for each fluorochrome-conjugated mouse Ig κ antibody to be used was labeled. 100 μ L of staining buffer was added to each tube followed by 1 full drop of previously vortexed BD CompBeads Anti-Mouse Ig κ and 1 drop of previously vortexed BD CompBeads Negative Control. 5 μ L of each antibody was then added to the appropriate tube and immediately vortexed. Tubes were incubated in the dark at room temperature for 20 minutes, washed twice with staining buffer and then acquired using the BD Canto II flow cytometer (BD Biosciences, UK).

2.5.2 Staining of cell lines and primary MM plasma cells

Surface staining of MM cell lines was performed to determine the expression of the surface markers described in Table 2-11. Panel A was used to characterise MM cells and confirm their identity on the basis of results present in the literature (Drexler and Matsuo 2000). Panel B was used to investigate transgene expression following infection with lentiviral vectors. Panel C was used to evaluate changes in the expression of CD80 ligands in a sorted CD80⁺ U266 cell population. Cells were counted and 0.5 $\times 10^6$ to 1 $\times 10^6$ cells were washed with staining buffer, resuspended in the residual volume of buffer and directly labeled with fluorochrome-conjugated monoclonal antibodies (mAbs) for 30 minutes in the dark at room temperature. Cells were then washed twice, resuspended in 250 μ L of staining buffer and then acquired using the BD Canto II flow cytometer (BD Biosciences, UK). A minimum of 10000 events was acquired each time.

Table 2-11 MM cells antibody stain mix.

Panel	Antibody	Clone	Fluorochrome	Manufacturer
A	CD138	MI15	Pacific Blue	BD Biosciences, UK
	CD28	CD28.2	FITC	BD Biosciences, UK
	CD56	B159	PerCPCy5.5	BD Biosciences, UK
	CD20	L27	Pacific Blue	BD Biosciences, UK
	CD19	HIB19	APC	BD Biosciences, UK
	HLA-ABC	DX17	PE	BD Biosciences, UK
	HLA-DR	G46-6	APC-H7	BD Biosciences, UK
B	CD80	L307.4	PE	BD Biosciences, UK
	Isotype control for CD80	MOPC-21	PE	BD Biosciences, UK
C	PD-L1	MIH1	PE	BD Biosciences, UK
	CD28	CD28.2	FITC	BD Biosciences, UK

2.5.3 Staining of PBMCs and NK cells

Cells were washed, resuspended in DPBS at a concentration of 0.5×10^6 to 1×10^6 cells/mL and aliquoted in polypropylene tubes. Each tube then received the appropriate antibody mixture according to the panel investigated (see 2-11) and cells were incubated in the dark at room temperature for 30 minutes. Anti-CD14, anti-CD19 and anti-CD138 antibodies were added in order to exclude B cells, monocytes and MM cells during the gating strategy.

2.5.3.1 PBMCs

The antibody panel described in Table 2-12 was created and optimised to investigate NK and T cell proliferation following co-culture with unmodified and genetically modified cells. NKG2D was added as a shared activation marker between NK and T cells. The total numbers of NK and T cells were determined by multiplying the number of stained lymphocytes (defined as trypan blue negative cells) by the percentage of NK or T cells (calculated using results from flow cytometry staining and expressed as percentage of lymphocytes).

Table 2-12 PBMCs proliferation antibody stain mix.

Panel	Antibody	Clone	Fluorochrome	Manufacturer
PBMCs proliferation	CD138	MI15	Pacific Blue	BD Biosciences, UK
	CD19	HIB19	Pacific Blue	BD Biosciences, UK
	CD14	MΦPg	Pacific Blue	BD Biosciences, UK
	CD56	B159	PerCPCy5.5	BD Biosciences, UK
	CD3	UCHT1	Pacific Orange	BD Biosciences, UK
	CD4	RPA-T4	FITC	BD Biosciences, UK
	CD8	RPA-78	PeCy7	BD Biosciences, UK
	NKG2D	1D11	APC	BD Biosciences, UK

2.5.3.2 NK cells and T cells

The antibody panels described in Table 2-13 and 2-14 were created and optimised to investigate NK cell activation and inhibition and T cell activation following co-culture with unmodified and genetically modified cells. Live/Dead stain (Invitrogen, UK) discrimination marker was added in order to avoid false positive signals caused by the non-specific binding of antibodies to dead cells. Activation markers were included on the basis of results present in the recent literature as reviewed in Chapter 1.

Table 2-13 NK cell activation antibody stain mix.

Panel	Antibody	Clone	Fluorochrome	Manufacturer
A	CD138	MI15	Pacific Blue	BD Biosciences, UK
	CD19	HIB19	Pacific Blue	BD Biosciences, UK
	CD14	MΦPg	Pacific Blue	BD Biosciences, UK
	CD56	B159	PerCPCy5.5	BD Biosciences, UK
	CD3	SK7	PeCy7	BD Biosciences, UK
	NKG2D	1D11	APC	BD Biosciences, UK
	DNAM-1	DX11	FITC	BD Biosciences, UK
	NKp30	P30-15	PE	BD Biosciences, UK
	Live/Dead stain	n/a	Aqua (V500)	Invitrogen
B	CD138	MI15	Pacific Blue	BD Biosciences, UK
	CD19	HIB19	Pacific Blue	BD Biosciences, UK
	CD14	MΦPg	Pacific Blue	BD Biosciences, UK
	CD56	B159	PerCPCy5.5	BD Biosciences, UK
	CD3	SK7	PeCy7	BD Biosciences, UK
	NKp46	9E2/NKp46	APC	BD Biosciences, UK
	NKp44	P44/81	PE	BD Biosciences, UK
	CD69	L78	FITC	BD Biosciences, UK
	Live/Dead stain	n/a	Aqua (V500)	Invitrogen

Table 2-14 T cell activation antibody stain mix.

Panel	Antibody	Clone	Fluorochrome	Manufacturer
T cells	CD138	MI15	Pacific Blue	BD Biosciences, UK
	CD56	B159	PerCPCy5.5	BD Biosciences, UK
	CD3	UCHT1	Pacific Orange	BD Biosciences, UK
	CD8	RPA-78	PeCy7	BD Biosciences, UK
	CD154 PE	TRAP1	PE	BD Biosciences, UK
	CD69 FITC	L78	FITC	BD Biosciences, UK
	HLA-DR	G46-6	APC-H7	BD Biosciences, UK

2.5.4 Detection of NK cell cytotoxicity

NK cell cytolytic activity was measured by different flow-cytometry based techniques.

2.5.4.1 Non-radioactive killing assay

A non-radioactive flow cytometry-based method was used as an alternative to chromium-release assay, to detect NK cell-induced apoptosis. This technique is based on the measurement of naked DNA in tumour cells after a 4 hour incubation with different PBMCs or purified NK cells as effector cells, at multiple effector to target ratios.

In order to distinguish target cells from effector cells, the former were washed twice in DPBS and pre-labeled with 1 μ M of carboxyfluorescein succinimidyl ester (CFSE) (Sigma, UK). CFSE crosses intact cell membranes and, once inside the cells, is cleaved by intracellular esterases and retained inside the cells, where it cross-links to intracellular proteins. The resulting molecules have a peak excitation at 494 nm and peak emission at 521 nm, normally measured on the FITC channel by flow cytometry. After an eight minute incubation with CFSE, cells are washed twice in complete RPMI1640 medium and frozen at 2×10^6 cells per aliquot as described in 2.1.4.1. On the day of the assay, these cells are thawed as described in 2.1.4.1 and resuspended in complete RPMI-1640 medium supplemented with 60 IU/mL of IL-2 at 2×10^4 cells/mL. Effector cells are washed twice, resuspended in X-VIVO 15 complete medium at 1×10^6 cells/mL (when PBMCs were used as effector cells) and 1×10^5 cells/mL (when NK cells were used as effector cells).

Effector and target cells were seeded in triplicates in a U-bottom 96 well plate. Each well received an appropriate volume of cell suspension in order to obtain effector to target ratios of 50 to 1, 25 to 1 and 12.5 to 1, when PBMCs were used as effectors. Instead, when NK cells were used as effectors, 5 to 1, 2.5 to 1 and 1.25 to 1 effector to target ratios were used, to reflect the NK cells proportion within the PBMCs population (Cooper *et al.* 2001). An appropriate volume of X-VIVO 15 complete medium was also added to each well to achieve a final volume of 200 μ L. Negative control wells were

seeded in order to quantify spontaneous target cell death by aliquoting 100 µL of target cell suspension and 100 µL of X-VIVO 15 complete medium supplemented with IL-2, 60 IU/mL. The seeded cells were then gently pelleted by centrifuging the plate at 120 x g with low brake and incubated in humidified 37 °C, 5% CO₂ incubators for four hours.

At the end of the incubation, cells were pelleted by centrifuging the plate at 300 x g with low brake, the supernatant was discarded and cells were resuspended in 100 µL of DPBS plus 4µL of 7-aminoactinomycin D (7-AAD; BD Biosciences, UK) per well. This fluorescent chemical compound is able to enter only the compromised membranes of dead cells and, once inside the cells, it intercalates in double stranded DNA. 7-AAD has a peak excitation at 488 nm and peak emission at 650 nm, normally measured on the APC or PerCP channel and characterised by a minimal overlap with FITC (CFSE). A minimum of 20000 CFSE positive events were acquired using BD Canto II flow cytometer (BD Biosciences, UK) and NK cell cytolytic activity was calculated according to the following formula

$$\% \text{ of lysis} = \frac{CFSE^{+}7AAD^{+} cells_{effectors+target\ cells} - CFSE^{+}7AAD^{+} cells_{target\ cells\ alone}}{100}$$

2.5.4.2 NK cell degranulation and IFN-γ production

Another method to investigate NK cell activation is based on the measurement of CD107a expression and IFN-γ production by NK cells following co-culture with target cells.

CD107a, also known as lysosomal-associated membrane protein (LAMP-1), is a receptor that lays in the luminal membrane of pre-formed cytolytic granules in NK cells (Winchester 2001). When the granules are released following NK cell activation, they fuse with the NK cell membrane where CD107a can then be detected on the outer cell surface. Recently, CD107a has been described as a marker for the activation of CD8⁺ T-cells and NK cells (Alter *et al.* 2004). As a strong relationship exists between the information provided by the killing assay (chromium release assay and 7-AAD assay) and CD107a expression, an assay to evaluate NK cell degranulation was developed with the aim of confirming the results obtained using the killing assay and to further

characterise the level of activation of the effector population. IFN- γ release was also included as an additional activation marker, as it is significantly associated with CD107a expression (Alter *et al.* 2004).

Effector and target cells were resuspended at 5×10^6 cells/mL in complete X-VIVO 15 and RPMI1640 medium supplemented with 60 IU/mL of IL-2 respectively. Based on preliminary results using K562 cells as targets (Figure 2-7), an effector to target cell ratio of 1 to 1 was chosen for subsequent experiments.

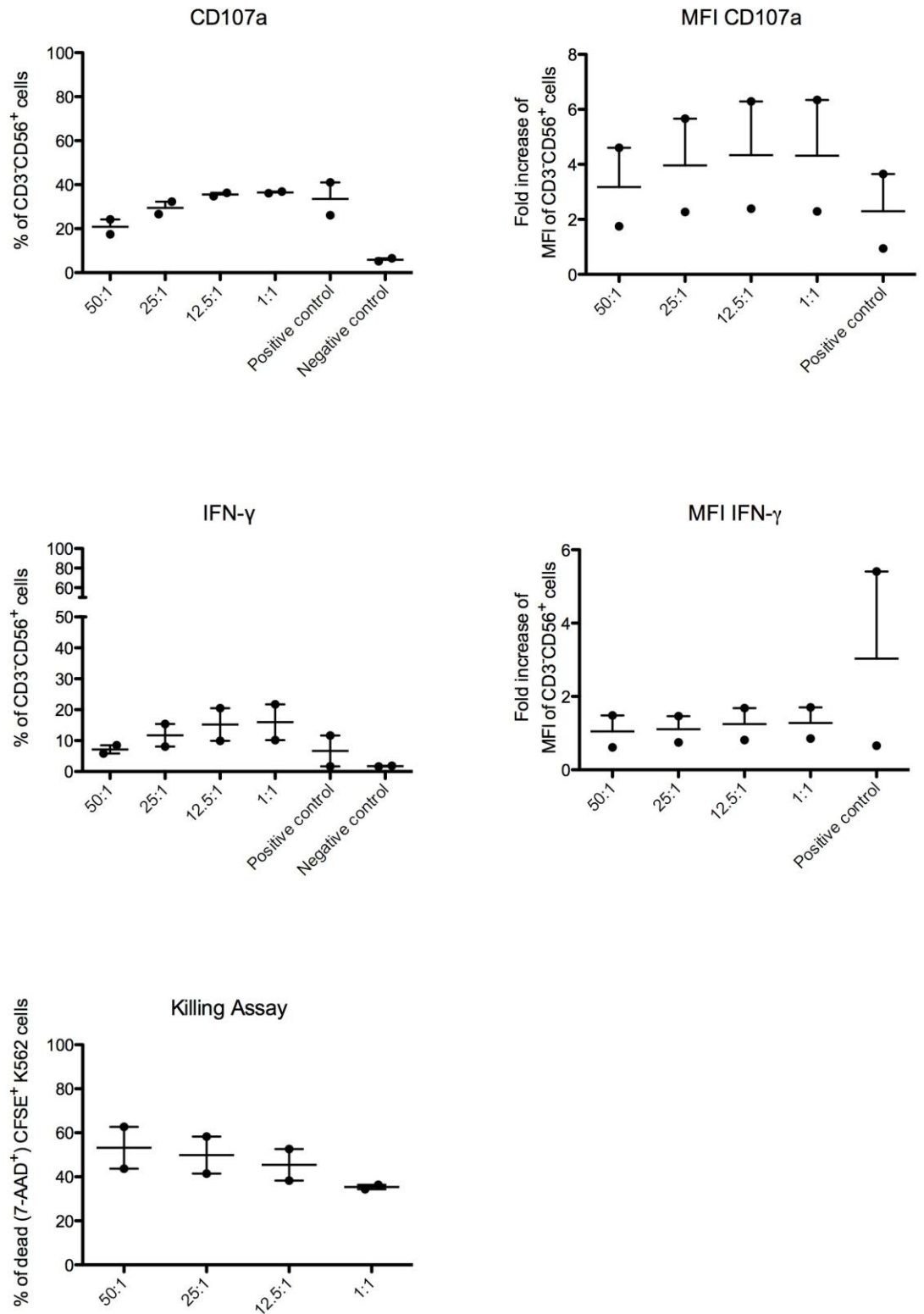


Figure 2-7 Optimisation of CD107a/intracellular IFN- γ detection assay. Assays were performed as described in 2.5.4.1 and 2.5.4.2. The X axis indicate the effector (PBMCs) to target (K562) ratio and the culture conditions. The graphs represent the median and range obtained from 2 healthy donors.

Each well of the 96 wells flat-bottom plate received 100 μ L of effector cells and 100 μ L of target cells. In order to quantify spontaneous NK cell degranulation and IFN- γ release, negative control wells were seeded by aliquoting 100 μ L of effector cell suspension and 100 μ L of RPMI-1640 complete medium supplemented with 60 IU/mL IL-2. Positive control wells were also set by aliquoting 100 μ L of effector cell suspension and 100 μ L of RPMI1640 complete medium supplemented with 60 IU/mL IL-2, phorbol 12-myristate 13-acetate (PMA) (10 μ g/mL) and ionomycin (1 μ g/mL) (both Sigma, UK). Each condition was performed in triplicate and each well received 2 μ L of anti-CD107a antibody. Cells were then gently pelleted by centrifuging the plate at 120 x g with low brake and incubated in humidified 37 °C, 5% CO₂ incubators for one hour.

At the end of this incubation time each well received an appropriate volume of monensin (BioLegend, USA) to achieve a final concentration of 2 μ M. Monensin is a protein-transport inhibitor commonly used to enhance intracellular cytokine staining signals and inhibiting protein recycling, by blocking transport processes at the Golgi level and neutralising the acidic pH of endosomes (Mollenhauer *et al.* 1990; Chan and Kaur 2007). Plated cells were then cultured for a further five hours in humidified 37 °C, 5% CO₂ incubators. Cells were then resuspended and transferred to a V bottom well plate, which is more suitable for the following staining and washing steps. Plated cells were centrifuged at 300 x g for 5 minutes with maximum brake and 150 μ L of the supernatant was discarded.

Cell pellets were resuspended and stained according to Table 2-15.

Table 2-15 CD107a-IFN- γ antibody staining mix

Antibody	Clone	Fluorochrome	Manufacturer
CD138	MI15	Pacific Blue	BD Biosciences, UK
CD19	H1B19	Pacific Blue	BD Biosciences, UK
CD14	MΦPg	Pacific Blue	BD Biosciences, UK
CD56	B159	PE	BD Biosciences, UK
CD3	SK7	PeCy7	BD Biosciences, UK
CD107a	H4A3	APC	BD Biosciences, UK
IFN- γ	4S.B3	FITC	BD Biosciences, UK

After a 30 minute incubation at room temperature, cells were washed twice with 200 μ L of staining buffer, resuspended in 100 μ L of Fix Buffer (BD Biosciences, UK), mixed well and incubated for 20 minutes at room temperature. After two further washes with 200 μ L of staining buffer, cells were resuspended in 100 μ L of perm/wash buffer (1:10 diluted with dH₂O; BD Biosciences, UK), mixed well and incubated for 15 minutes at room temperature. The plate was then centrifuged at 300 x g for 5 minutes with maximum brake, the supernatant was discarded and cells were resuspended with FITC-labelled IFN- γ diluted in 30 mL of perm/wash buffer. After a 30 minute incubation, cells were washed twice with perm/wash buffer, resuspended in 200 μ L of staining buffer and acquired using the BD Canto II flow cytometer (BD Biosciences, UK). A minimum of 30000 events was recorded.

2.6 ELISA

ELISA (Enzyme-Linked Immunosorbent Assay) is a widely used biochemical technique for the detection of a specific antigen in a sample. For this study, we used a variant of this technique, called sandwich ELISA, which utilises two antigen-specific antibodies: a capture antibody bound to a solid phase and an enzyme-linked detection antibody.

A microtiter plate (R&D System, UK) was coated with the capture antibody, sealed with the provided plate cover and incubated overnight at room temperature. In the case of Granzyme B ELISA, a ready-to-use pre-coated plate was employed. Subsequently, each well was aspirated and washed three times with ELISA wash buffer (DPBS with 0.05% Tween; Sigma, UK) using a squirt bottle, and after the last wash, any remaining wash buffer was removed by inverting and blotting the plate against clean paper towels. Each well then received 100 μ L of blocking buffer (DPBS 1% BSA, all Sigma, UK) and was incubated at room temperature for a minimum of 1 hour. Multiple sample dilutions were applied in triplicates to the plate and incubated for 2 hours to allow the antigen in the sample to bind to the capture antibody. As part of the assay quality control and assay validation, a standard curve was prepared by aliquoting serial dilutions of a known concentration of the protein standard in duplicates, and negative control wells containing the sample diluent were also set up. After the incubation, unbound compounds were removed by performing the washing procedure previously described. 100 μ L of diluted detection antibody were added to each well and incubated for 2 hours to allow the antibody to bind to a second epitope on the antigen. Unbound antibodies were removed by a washing step. Subsequently, 100 μ L of HRP-Conjugated Streptavidin were added to the wells. This compound consists of streptavidin protein, which binds to the biotinylated detection antibody, and is covalently conjugated to the horseradish peroxidase (HRP) enzyme. After a 20 minute incubation and the following washing step, 100 μ L of the chromogenic substrate 3',3',5,5' Tetramethylbenzidine (or TMB) was

added to each well. This molecule is oxidated by the HRP causing the solution to take on a blue colour. After 10 to 20 minute incubation, the reaction was stopped by adding 50 μ L of 1N sulphuric acid, which offers a yellow end product and the plate was read at 450 nm using a spectrophotometer (BioTeck, UK).

Data were analysed using the Gene5 Data Analysis Software (BioTeck, UK). Through this program, it was possible to generate a three or four parameter logistic curve fit, which plots the mean absorbance for each standard on the y axis against the known concentration present in the well on the x axis (Figure 2-8). The coefficient of determination (R^2) was used as an indication of the inter-assay reproducibility, with values from 0.98 to 1 representing the best result.

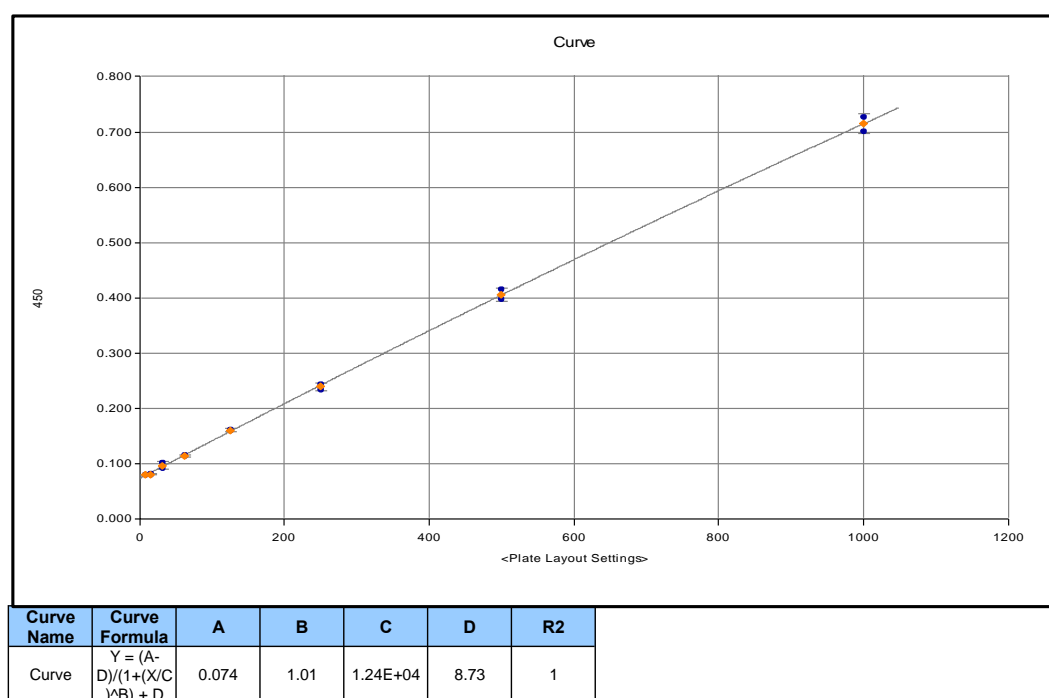


Figure 2-8 ELISA standard curve fit.

The standard curve was drawn by plotting the mean absorbance at 450 nm against the known protein concentration present in the standard dilutions as indicated on the plate layout. The table below the graph reports the curve formula used for the fitting and the R^2 value.

The concentration of the sample of interest was then determined by interpolation using the curve described above. This value was then subtracted of the antigen concentration detected in the negative control wells (background) and, when appropriate, multiplied by the dilution factor to obtain the protein concentration per mL of culture.

2.7 MTT assay

The MTT assay is a non-radioactive cell proliferation assay (Promega, UK) based on the cellular conversion of a tetrazolium salt into a formazan product, which is then detected using a 96-well plate reader.

Cells were washed twice in complete fresh medium and centrifuged at 300 x g for 5 minutes with maximum brake. Cells were resuspended in complete medium and cell numbers and viability were determined. Cell concentration was adjusted to 1×10^6 cells/mL and each well of a 96-well plate received 100 μ L of cell suspension. The plate was incubated in humidified 37°C, 5% CO₂ incubators. After 48 hours, 15 μ L of the dye solution was added to each well and the plate was incubated in humidified 37°C, 5% CO₂ incubators. During this time live cells convert the tetrazolium component of the dye solution into a formazan product. After 4 hours, 100 μ L of the Solubilisation Solution/Stop mix were added to each well to solubilise the formazan product and its absorbance at 570 nm was recorded using a spectrophotometer (BioTek, UK).

Data were analysed using the Gene5 Data Analysis Software (BioTeck, USA).

2.8 Statistical analysis

Computer software GraphPad Prism® (version 5.0, La Jolla, California, USA) was used for the statistical calculations and to plot graphs. Data are visually presented as mean with standard deviation, or median with range, or grand median, which represents the combined samples median.

The distribution of the data was determined using the SPSS software (IBM). Due to the non-normal distribution of data, statistical significance was calculated by comparing the medians, using the non-parametric matched paired analysis Wilcoxon signed rank-test, when considering two parameters, and the Friedman test when performing a One-way Anova analysis on more than two parameters. Results are presented as P values, with a 95% confidence interval. Statistical significance was set at $P < 0.05$.

Chapter 3 Results

3.1 Multiple myeloma-mediated inhibition of NK cells

3.1.1 Introduction

Like many other malignancies, MM is associated with a profound impairment of immune functions (see 1.3.3), allowing the tumour to escape immune surveillance. So far, studies investigating this phenomenon have mainly addressed the impact of cytokines and other soluble immunomodulatory factors on the adaptive arm of the immune system (Godfrey and Benson 2012). However, in the past 20 years, numerous works have also highlighted the fundamental contribution of NK cells to the control of malignant plasma cell clones and have started to investigate the mechanisms used by malignant MM plasma cells to escape NK cell recognition and killing (as described in Chapter 1.3).

The work described in this chapter aims to further elucidate the biology underlying MM-induced inhibition of NK cells and to test the hypothesis that MM cells are able to specifically inhibit NK cell functions and that this inhibition is achieved through contact-dependent interactions between NK and MM cells.

These hypotheses are supported by recent studies (reviewed in 1.3.2), which have showed the modulation of the receptor-ligand interactions that mediate NK cell surveillance and cytotoxicity. After findings obtained in various tumour setting, El-Sherbiny and colleagues (El-Sherbiny *et al.* 2007) described, through immune-phenotypic analysis and the use of monoclonal antibodies during cytotoxic

assays, the requirement for DNAM-1, NKG2D and NKp46 in the NK cell-mediated killing of MM cells, using both healthy donor and patient samples. Subsequently, a reduced expression of NKG2D in MM patients was shown and the use of co-culture experiments suggested a casual link to cell-to-cell interactions, rather than to the release of soluble factors by MM cells (von Lilienfeld-Toal *et al.* 2010). This finding was then extended to other NK activating receptors and was confirmed also in other malignancies such as melanoma (Balsamo *et al.* 2009; Pietra *et al.* 2012), breast cancer (Mamessier *et al.* 2011), cervical cancer (Garcia-Iglesias *et al.* 2009) and acute myeloid leukaemia (Sanchez *et al.* 2011).

On the basis of these findings, an *in vitro* model was optimised in order to investigate MM-induced inhibition of NK cells. Transwell co-cultures were employed in order to elucidate the role of cytokines and other soluble factors versus contact-mediated interactions. Isolated NK cells were used to investigate the direct effect of MM cells on NK cell phenotype and cytolytic activity.

The optimised *in vitro* system was able to reproduce the functional inhibition of NK cells and the decreased expression of NKG2D and NKp30, which are characteristic of NK cells isolated from MM patients (El-Sherbiny *et al.* 2007; von Lilienfeld-Toal *et al.* 2010; Pietra *et al.* 2012). The data highlighted the importance of receptor-ligand interactions in MM-induced immunosuppression as, when cell-to-cell contact was prevented, NK cells retained the phenotypic characteristics and activity of NK cells isolated from healthy donors. Finally, the results from the co-culture of isolated NK cells with the MM cell line U266 showed that MM-induced impairment of NK cell function is directed specifically on NK cells and is not dependent on the presence of other peripheral blood components.

3.1.2 Results

3.1.2.1 MM cell lines inhibit NK cell activity

To investigate whether MM cells could affect NK cell activity *in vitro*, PBMCs from healthy donors were cultured either with medium alone or in the presence of three different MM cell lines (KMS12PE, RPMI8226, and U266). NK cells were subsequently isolated and analysed for their cytolytic activity against the Natural-Killer sensitive cell line K562.

As shown in Figure 3-1, preliminary assays showed that the cytolytic activity of NK cells isolated from PBMCs co-cultured with MM cell lines was variably impaired. The MM cell lines U266 and RPMI8226 caused a greater than two-fold reduction in the cytolytic activity of NK cells, from 54.9% 7-AAD⁺CFSE⁺ K562 cells to median of 25.2% and 25.45% 7-AAD⁺CFSE⁺ K562 cells respectively ($P \leq 0.01$ and $P \leq 0.05$). NK cytolytic activity after co-culture with KMS12PE cells did not show a statistical significance difference compared to that of healthy donor PBMCs (median of 47.9% versus 54.9% 7-AAD⁺CFSE⁺ K562).

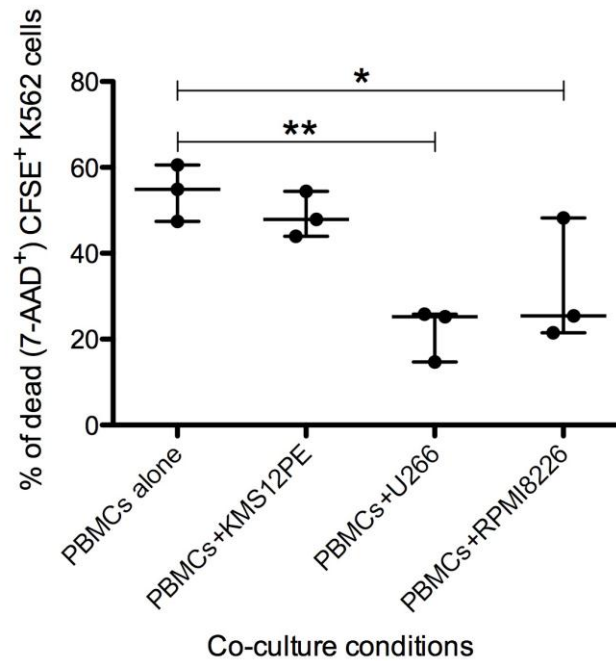


Figure 3-1 Impaired cytolytic activity of NK cells following co-culture with MM cell lines. PBMCs from three healthy donors were cultured for 24 hours either in complete medium alone or in the presence of three different MM cell lines (KMS12PE, RPMI8226, U266). Purified NK cells (as described in 2.4.1) were tested for cytolytic activity against K562 target cells at an effector to target ratio of 5 to 1. Results represent the percentage of lysis mediated by NK cells cultured in the indicated conditions. Data are represented as the median and range obtained from three independent experiments. * $P \leq 0.05$; **, $P \leq 0.01$

Additionally, a concurrent significant downregulation in the expression of the activating receptor NKG2D on the stimulated NK cells was observed (Figure 3-2). In fact, when incubated with U266 and RPMI8226 MM cells, the percentage of NK cells expressing NKG2D decreased from a median of 73.7% when considering PBMCs cultured in complete medium to a median of 31.2% and 51.4% for PBMCs co-cultured with U266 and RPMI8226 cells respectively ($P \leq 0.01$ and $P \leq 0.05$). The reduction in NKG2D positive cells after co-culture with KMS12PE did not reach statistical significance.

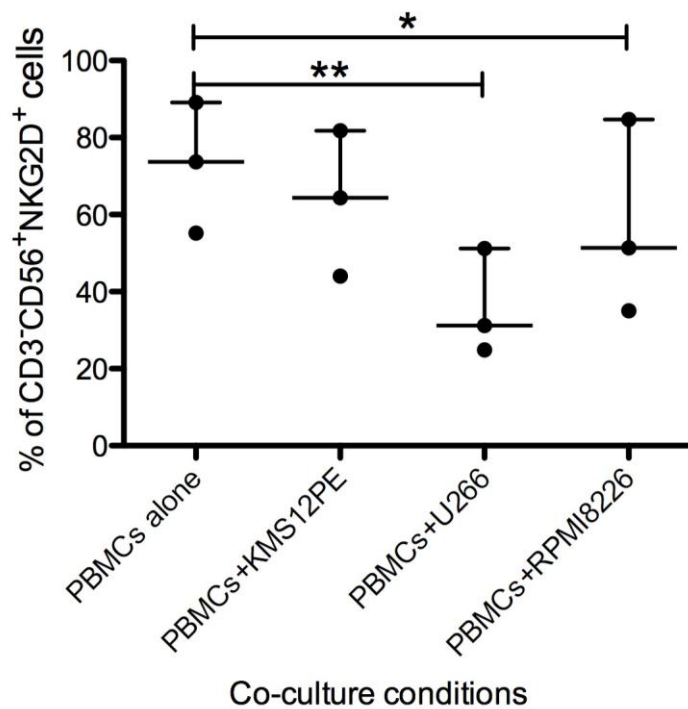


Figure 3-2 Effect of MM cell lines on the expression of the activating NK receptor NKG2D. PBMCs from three healthy donors were cultured as described in Figure 3-1. NK cells were purified and the expression of NKG2D was analysed by flow cytometry. Data are represented as median and range obtained from three independent experiments. * $P \leq 0.05$; **, $P \leq 0.01$.

The U266 cell line was chosen as reference cell line for subsequent assays on the basis of its ability to consistently induce a profound inhibition of NK cell activity and downregulation of NKG2D expression. The RPMI8226 cell line, despite producing similar results, was not considered due to its expression of CD56 and low expression of CD138 (as described in 2.1.2.2), which make their depletion for subsequent assays problematic.

The downregulation of NKG2D expression was used as a marker to determine the best incubation time to study the MM-induced inhibition of NK cells. As allo-reactions of T cells against U266 cells may occur during the co-culture, it was decided to screen time points up to 72 hours.

Additionally, to test whether the impairment of NK cell activity was specific to MM cells, an experiment was designed to compare their inhibitory ability with that of plasma B cells from healthy donors. However, as the number of circulating plasma cells is very limited (Horst *et al.* 2002), it was decided to use allogeneic CD20⁺ B cells, which belong to the same lineage as MM cells, as a control.

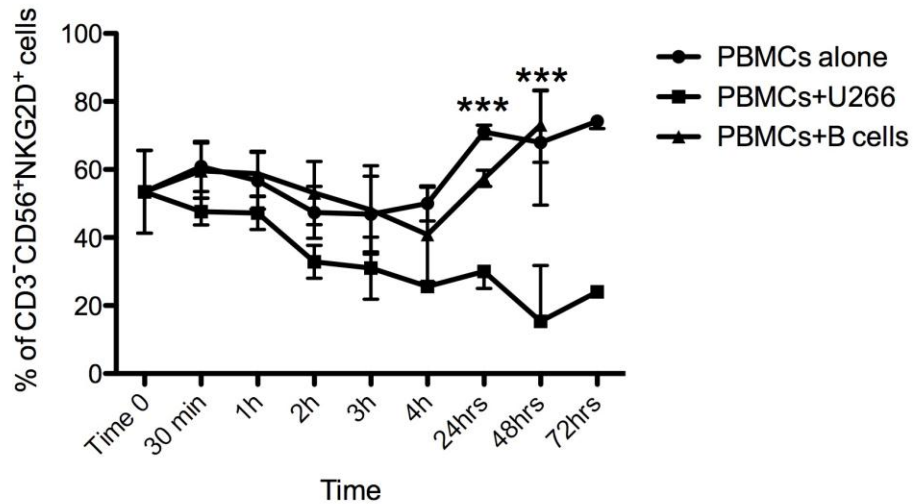


Figure 3-3 Effect of incubation time on the expression of NKG2D on NK cells following incubation with the MM cell line U266.

PBMCs were cultured either in complete medium alone or in the presence of the MM cell line U266 or allogeneic CD20⁺ B cells. The expression of NKG2D on NK cells was analysed by flow cytometry at the time points indicated. Results represent the median with range obtained from two independent experiments. *** $P \leq 0.001$.

The results shown in Figure 3-3 suggest 24 hours and 48 hours as suitable time points to study MM-induced inhibition of NK cells. However, in order to be consistent with previous studies (von Lilienfeld-Toal *et al.* 2010), 24 hours was chosen for subsequent assays. Furthermore, this inhibition can be considered specific to the MM model as it was not observed when using B cells from healthy donors as a negative control.

3.1.2.2 The MM-induced inhibition of NK cells activity is not caused by the exhaustion of NK cells

NK cell impairment can be caused by various mechanisms, including extensive degranulation by NK cells in the presence of its target cells, which may heavily reduce their availability for subsequent lysis processes. Therefore, the ability of NK cells to release granzyme B while in co-culture with MM cells was measured. Granzyme B was chosen as it is considered to be a major effector of target-cell killing by NK cells (Rousalova and Krepela 2010). K562 cells, known to trigger the release of Granzyme B from NK cells, were used as a positive control.

As demonstrated in figure 3-4, after a 24 hour co-culture with the MM cell line U266, the levels of Granzyme B in the culture medium were still increasing, demonstrating that NK cells had not been depleted of Granzyme B.

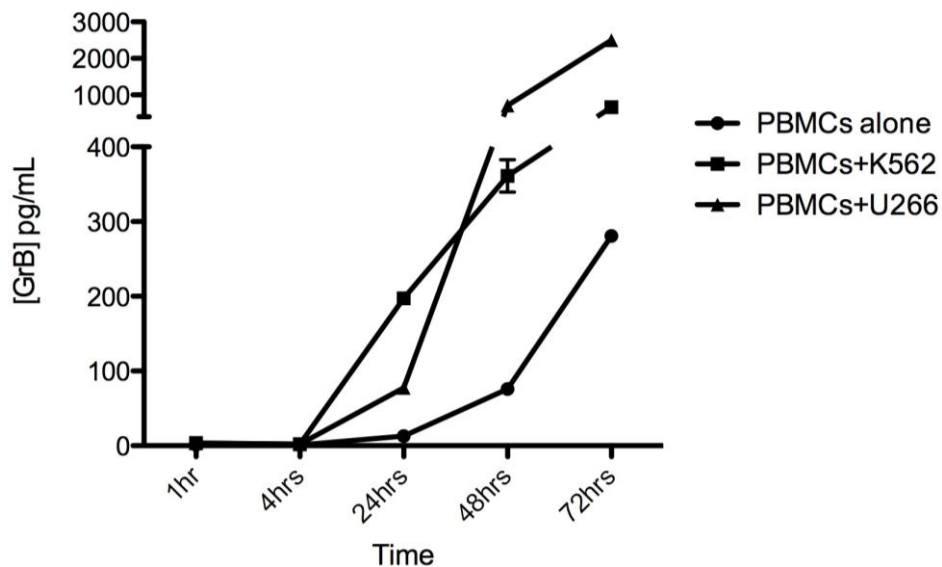


Figure 3-4 Granzyme B release from PBMCs cultured either in complete medium alone or in the presence of the MM cell line U266 or K562 cells. Supernatants were harvested at the indicated time points and tested for the presence of Granzyme B by ELISA assay. Results are a representative example (triplicates \pm standard deviation) of two independent experiments.

Therefore, inhibition of NK cell cytolytic activity is unlikely to be due to exhaustion of Granzyme B levels. It is also worth noting that the MM U266 cell line is able to induce a higher although slightly delayed Granzyme B release compared to K562 cells.

3.1.2.3 The MM cell line U266 is able to inhibit NK cell activity through contact-dependent interactions

Because a number of soluble and non-soluble factors are known to be involved in tumour-mediated suppressive effects on the immune system (Oppenheim *et al.* 2005; Kloss *et al.* 2008; von Lilienfeld-Toal *et al.* 2010), the mechanisms involved in MM-induced inhibition of NK cells was next examined.

As shown in figure 3-5, prevention of contact-dependent interactions between U266 cell and NK cells through the use of a transwell system blocked U266 cell-induced inhibition of NK cell cytolytic activity. When contact between PBMCs and U266 was

prevented, the percentage of K562 killing was, in fact, restored from a median of 16.2% to 55.2% ($P \leq 0.001$).

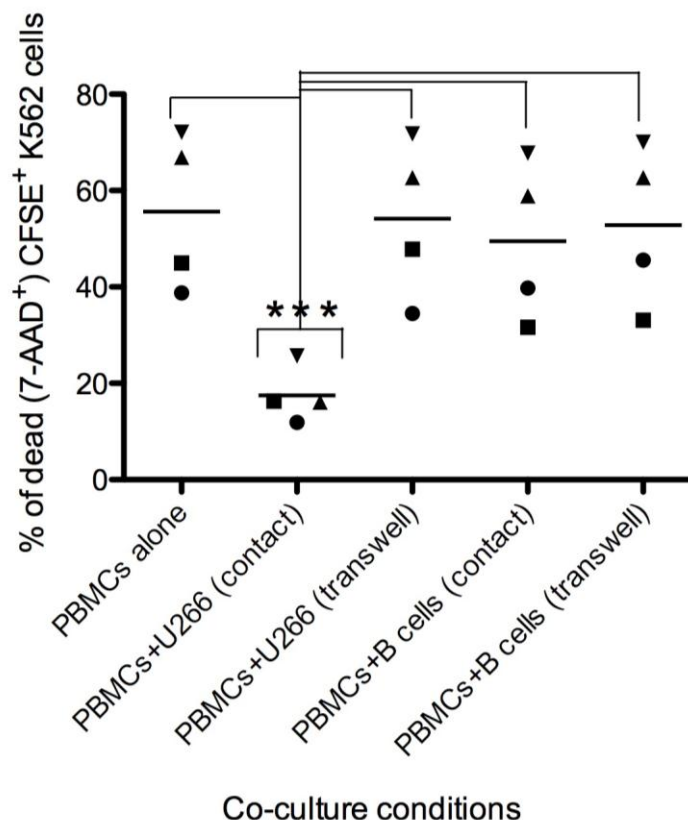


Figure 3-5 Contact-dependent inhibition of NK cells following co-culture with U266 MM cell line. Healthy donor PBMCs were cultured either in complete medium alone or in the presence of the MM cell line U266 or allogeneic CD20⁺ B cells. In one condition, contact-dependent interactions were prevented through the use of a transwell system. Data are represented as the median of purified NK cell cytolytic activity obtained from four independent experiments. *** $P \leq 0.001$.

3.1.2.4 MM cell-induced impairment of NK cells is associated with decreased expression of the activating receptors NKG2D, NKp30 and DNAM-1

The next aim was to examine whether the decrease in the cytolytic activity of NK cells was associated with changes in the extracellular expression of NK cell activating receptors other than NKG2D.

Figure 3-6 shows that, after co-culture with the MM cell line U266, NK cells displayed a decrease in the expression of NKG2D, NKp30 and DNAM-1, when compared with those isolated from PBMCs incubated in complete medium alone or in the presence of allogeneic B cells. Specifically, the percentage of NK cells expressing NKG2D decreased from a median of 74.35% to 22.5% ($p \leq 0.001$), whereas NKp30 was

down-regulated 1.6 fold (median of 77.85% compared to 47.15%; $p \leq 0.01$) and DNAM-1 expression went from a median of 85.85% to 60.7% ($p \leq 0.01$). Interestingly, the intensity of NKG2D expression on NK cells was reduced from a mean fluorescence intensity (MFI) of 2746 to 1592.5 ($p \leq 0.05$). Once again, this effect was specific to MM cells and required contact-dependent cell-to-cell interactions.

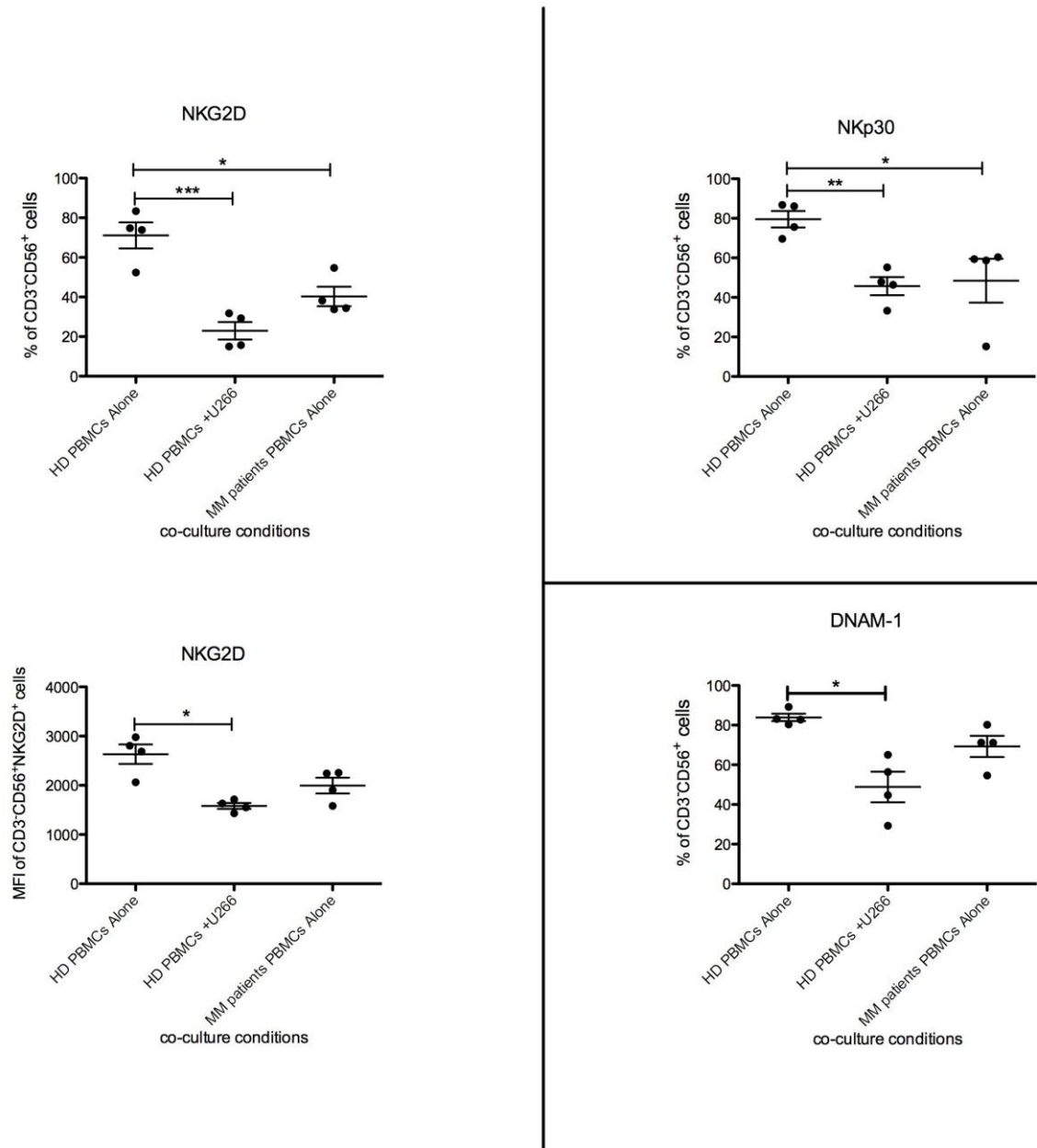


Figure 3-6 Effect of MM cell line U266 on the expression of activating NK receptors. Healthy donor PBMCs were cultured as described in Figure 3-5. Subsequently, the expression of NCR on NK cells was analysed by flow cytometry. Data are represented as the median of the expression of activating receptors obtained from four healthy donors. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.1.2.5 Primary NK cells isolated from MM patients display impaired cytolytic activity and an immune phenotype similar to healthy donor NK cells co-cultured with MM cell lines

Next, the phenotype and functional activity of NK cells from MM patients were compared to those of healthy donor PBMCs prior and following *in vitro* co-culture with MM cells.

As illustrated in figure 3-7 and confirming the existing literature (Matsuzaki *et al.* 1985; Ogmundsdottir 1988; Jurisic *et al.* 2007), NK cells from MM patients displayed a suppressed cytolytic activity comparable to that observed when healthy donor PBMCs were co-cultured with the MM cell line U266. In fact, the percentage of K562 cells killing decreased from a median of 63.7% when considering healthy donor PBMCs, to 16.17% and 20.36% 7-AAD⁺CFSE⁺K562 when using healthy donor NK cells co-cultured with MM cells or MM patient NK cells respectively ($p \leq 0.01$).

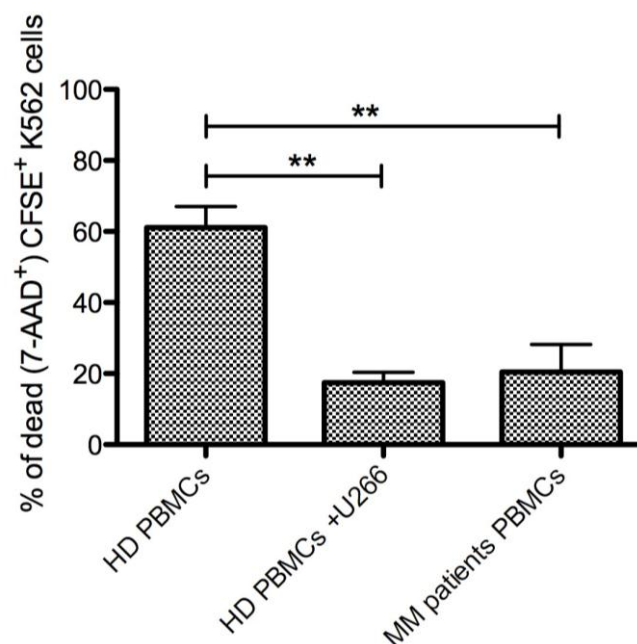


Figure 3-7 Impairment of NK cell activity by MM in healthy donor and MM patients. Healthy donor PBMCs were cultured for 24 hours either in complete medium alone or in the presence of the MM cell line U266. PBMCs from MM patients were cultured for 24 hours in complete medium. Data are represented as the median and range of purified NK cell cytolytic activity obtained from four independent experiments. ** $P \leq 0.01$.

Additionally, our data showed that NK cells from MM patients, as well as healthy donor NK cells co-cultured with MM cells lines, are characterised by a reduced

expression of NKG2D and NKp30. The expression of NKG2D decreased from a median of 74.35% in healthy donors to 22.5% in the presence of U266 cells ($P \leq 0.001$) and to 36.3% in MM patients PBMCs ($P \leq 0.05$). A similar effect was seen when considering NKG2D MFI, which was reduced from a median of 2374.5 in healthy donors to 1592.5 and 2072 in *in vitro* inhibited PBMCs ($P \leq 0.05$) and in MM patients PBMCs. The percentage of NK cells expressing NKp30 in HD was 77.85% but dropped to 47.15% ($P \leq 0.01$) after incubation with U266 cells and was measured around 59.05% ($P \leq 0.05$) in MM patients PBMCs. Although NK cells from MM patients showed a trend towards a reduction in DNAM-1 expression, the results obtained did not reach statistical significance (Fig. 3-8).

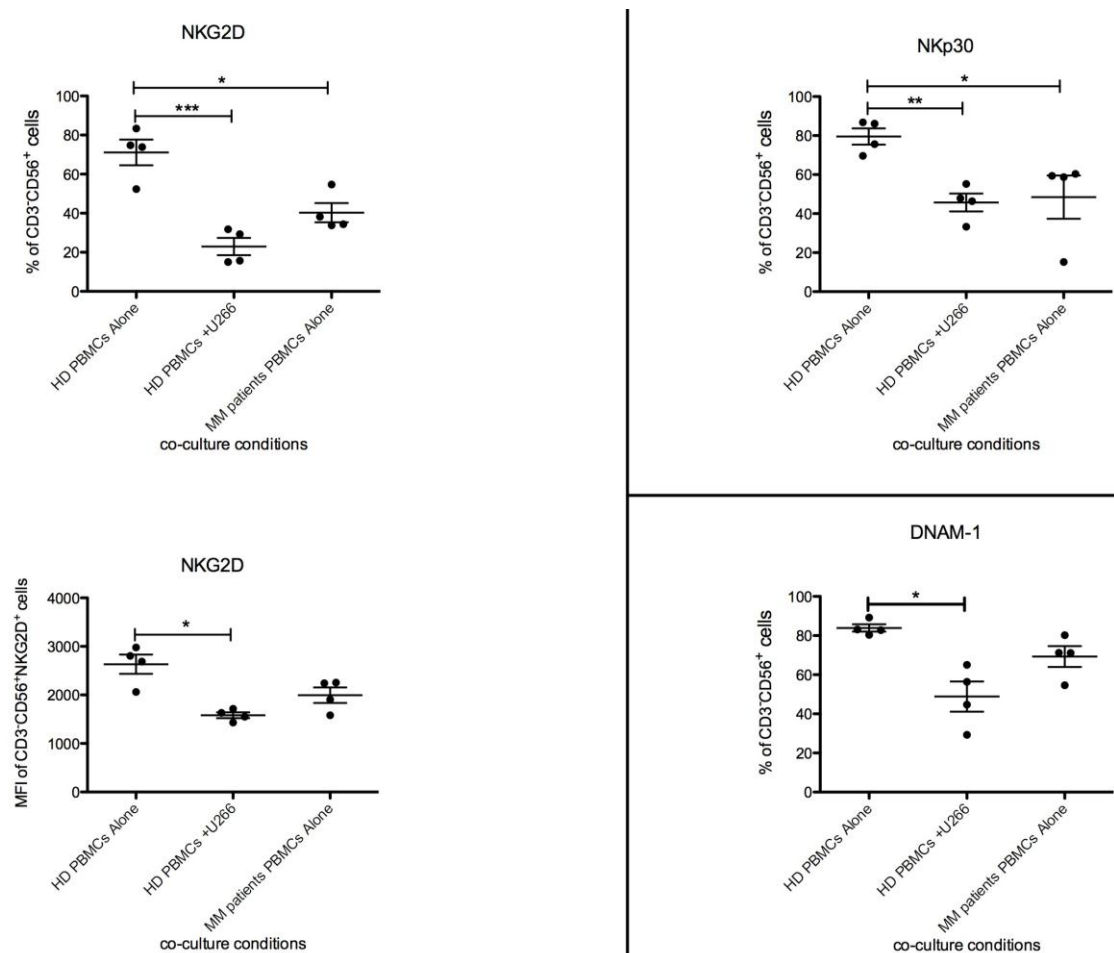


Figure 3-8 Effect of the MM cell line U266 on the expression of activating NK receptors. Cells were cultured as described in 3-7. Subsequently, the expression of NCR on NK cells was analysed by flow cytometry. Data are represented as the median of the expression of activating receptors obtained from four independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.1.2.6 MM-induced impairment of NK cell activity does not depend on the presence of other peripheral blood mononuclear cells

Tumour-induced immune suppression of NK cells may be due to direct effects on NK cells or, alternatively, may be mediated by other mononuclear cells present in the peripheral blood such as monocytes, macrophages and Treg (Kloss *et al.* 2008), which might have been activated by MM cells. To investigate this possibility, MM-induced inhibition on purified NK cells and on NK cells in the presence of other peripheral blood mononuclear cells was compared.

Similar results were obtained with regard to NK cell activation (Fig. 3-9 and 3-10) and NKG2D expression (Fig. 3-11), whether NK cells were cultured with PBMCs or isolated.

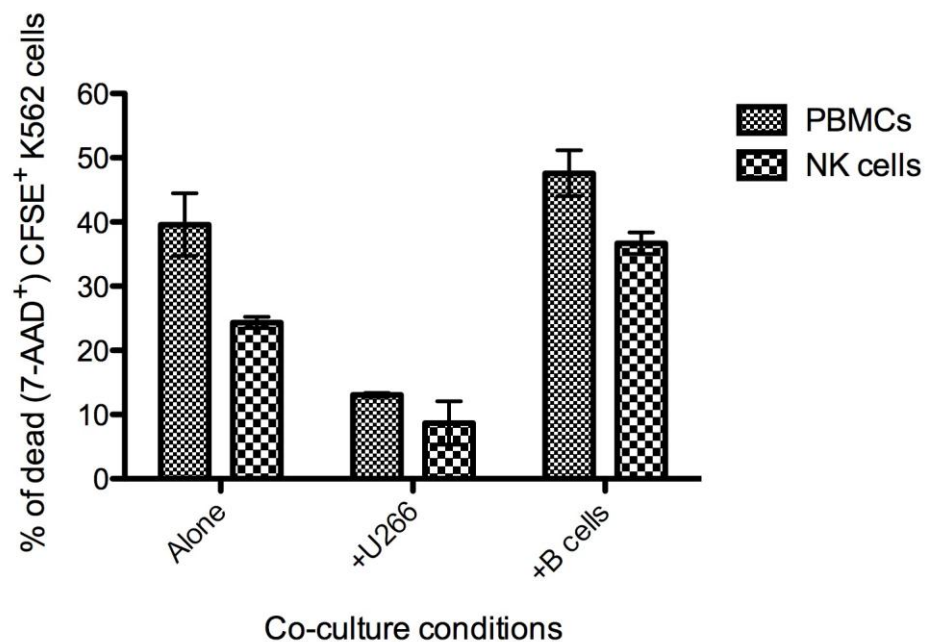


Figure 3-9 Effect of peripheral blood cell components on the MM cell-induced functional inhibition of NK cells.

Healthy donor PBMCs or purified NK cells were cultured for 24 hours either in complete medium alone or in the presence of the MM cell line U266 or allogeneic CD20⁺ B cells. Bars represent the median with range of the percentage of NK cell cytolytic activity, obtained from three healthy donors.

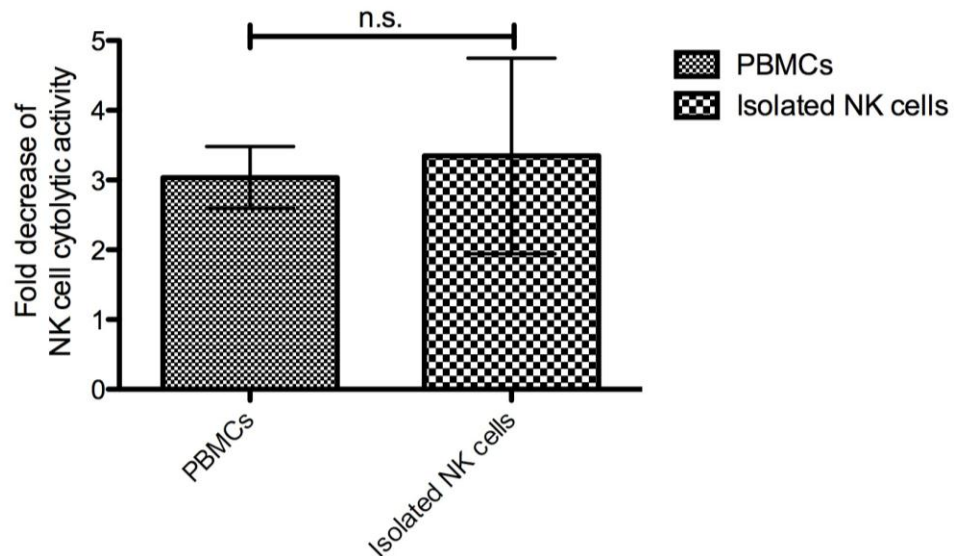


Figure 3-10 Effect of peripheral blood mononuclear cells on the MM cell-induced functional inhibition of NK cells.

Cells were cultured as described in Figure 3-9. Bars represent median with range of the fold decrease of NK cell cytolytic activity, obtained from three independent experiments. Results were normalised to those obtained from effector cells co-cultured alone.

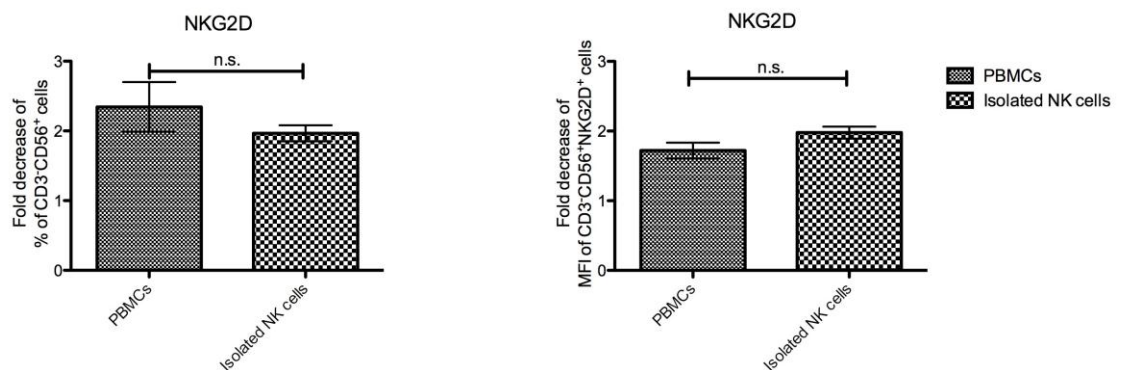


Figure 3-11 Effect of peripheral blood mononuclear cells on MM cell-induced downregulation of NKG2D.

Cells were cultured as described in Figure 3-9. Subsequently, the expression of NKG2D on NK cells was analysed by flow cytometry. Bars represent the median with range of the fold decrease of the percentages (left) and MFI (right) of NKG2D⁺ NK cells obtained from three independent experiments. Results were normalised to those obtained from effector cells co-cultured alone.

These data show that the presence or absence of peripheral blood components does not influence the MM-mediated impairment of NK cell function, thereby suggesting a direct inhibition on NK cells by MM cells.

3.1.3 Discussion

In vitro and *in vivo* cytolytic activity of NK cells against MM cells has been widely demonstrated (Godfrey and Benson 2012) (and reviewed in Chapter 1.4). However, NK cell-based immunotherapeutic strategies have resulted in limited clinical benefit, possibly reflecting the mechanisms that tumour cells have developed to avoid NK cell-mediated recognition and killing (Pietra *et al.* 2012).

The results described in this chapter show that a 24 hour co-culture of healthy donor PBMCs with three human MM cell lines was able, with various efficiencies, to impair NK cell cytolytic activity against the NK cell-sensitive cell line K562 and to induce the downregulation of NKG2D expression on NK cells. This is in agreement with the work published by von Lilienfeld-Toal and colleagues (von Lilienfeld-Toal *et al.* 2010). In addition, this effect was also specific to MM cells, as the co-culture with allogeneic CD20⁺ B cells, which belong to the same lineage of MM cells, was not able to suppress NK cell function. Finally, the levels of Granzyme B release following co-culture with U266 cells showed that the inhibition observed in NK cell cytolytic activity is not likely to be caused by NK cell exhaustion.

Recent studies using different malignancy models have shown that the downregulation of important NK cell receptors can be induced by persistent contact with tumour cells (Balsamo *et al.* 2009; Garcia-Iglesias *et al.* 2009; von Lilienfeld-Toal *et al.* 2010; Mamessier *et al.* 2011; Sanchez *et al.* 2011). Experiments performed using a transwell co-culture system demonstrated that both NK cell functional impairment and NKG2D downregulation, following co-culture with MM cells, require contact-dependent cell-to-cell interactions. In addition to the suppression of NKG2D levels, the present studies demonstrated the downregulation of other important activating receptors such as DNAM-1 and NKp30. These results are in agreement with previous works showing the expression of Natural Cytotoxic Receptor (NCR) ligands on MM cells (Soriani *et al.* 2009; von Lilienfeld-Toal *et al.* 2010) and are particularly relevant as DNAM-1 and NKG2D have been shown to be essential in the NK cell-mediated killing of MM (El-Sherbiny *et al.* 2007) and in the control of other malignancies (Balsamo *et al.* 2009; Carrega *et al.* 2009;

Mamessier *et al.* 2011; Sanchez *et al.* 2011). The importance of the downregulation of NKG2D on NK cell activation has also been extensively demonstrated in different *in vitro* (Spaggiari *et al.* 2008; Balsamo *et al.* 2009; Carrega *et al.* 2009; Mamessier *et al.* 2011; Pietra *et al.* 2012) and *in vivo* (Garcia-Iglesias *et al.* 2009; Sanchez *et al.* 2011) systems.

The potential relevance of these *in vitro* results was demonstrated by experiments showing that NK cells from MM patients display reduced cytolytic activity and lower cell surface expression of the activating receptors NKG2D and NKG2A similar to those characteristic of healthy donor NK cells co-cultured with MM cell lines. These results confirm findings published by von Lilienfeld-Toal and colleagues (von Lilienfeld-Toal *et al.* 2010) in the context of MM, and by Sanchez-Correa (Sanchez *et al.* 2011) in the context of other tumours, and may be indicative of immune selective mechanisms developed and shared by cancer cells. No statistically significant difference was, however, observed in the downregulation of DNAM-1 expression NK cells from MM patients. This could depend on the levels of expression of DNAM-1 ligands on MM cells, as suggested by von Lilienfeld-Toal and colleagues in the context of NKG2D (von Lilienfeld-Toal *et al.* 2010). Further studies are needed to test this hypothesis.

The role of peripheral blood components, such as monocytes, DCs and T cells, in the context of MM-induced impairment of NK cells was subsequently examined. The co-culture of isolated NK cells with MM cells resulted in a decrease of cytolytic activity and NKG2D expression comparable to that recorded when the whole PBMCs population was used as target of the inhibition. It is therefore possible to conclude that NK cell inhibition, as observed after a 24 hour co-culture with MM cells, is directed specifically to NK cells without any further contribution from other peripheral blood cellular or soluble component.

3.2 The production of high lentivirus titers for genetic modification of MM cells

3.2.1 Introduction

In view of the immunosuppressive effect shown in Chapter 3.1, new strategies should be developed to prevent and rescue myeloma-induced suppression of immune responses.

Autologous whole-cell vaccines may provide an attractive tool for the stimulation of immune responses against a wide range of tumour-associated antigens (Pratt *et al.* 2007; Rosenblatt and Avigan 2008; Rosenblatt *et al.* 2011). The use of genetically-modified tumour cells has, in fact, been shown to induce immunological responses against MM and other malignancies *in vitro* and *in vivo* (as reviewed in 1.4.2.4.2). In previous studies, MM cells have been successfully genetically-modified with adenoviral (Wen *et al.* 2001a; Ren *et al.* 2006) and retroviral (Tarte *et al.* 1999; Lu *et al.* 2007) vectors and shown to activate T cell stimulation. However, the problematic infection of primary tumour cells requires the use of high amounts of viral particles (Wen *et al.* 2001a; Ren *et al.* 2006) or the subsequent selection of the modified cells through the use of a selective marker and cell sorting (Tarte *et al.* 1999; Lu *et al.* 2007). This poses a challenge to the clinical application of these promising approaches as these long and stressful selection procedures may dramatically decrease the number of cells available for vaccination. Additionally, the use of great amounts of viral particles is restricted by the limits in the production of high titers of virus and by the potential cytotoxic effect of the lentiviral vector on target cells.

Therefore, a self-inactivating lentiviral vector (Chan *et al.* 2005a) was tested for its ability to genetically-modify human MM cell lines to stably express high levels of the immunoregulatory factors CD80 and IL-2.

An important feature contributing to this vector's efficiency is the unique advantage of lentiviral vectors to stably transduce non-dividing cells such as haematopoietic cells (Dropulic 2011). Additionally, the vesicular stomatitis virus G glycoprotein envelope

(VSV-G) used in this construct allows the transduction of a wide variety of mammalian cells (Dull *et al.* 1998) and remains infective after ultracentrifugation. This facilitates the concentration of high titers of virus and, consequently, the transduction of target cells.

Two similar vectors were used by Cignetti's group (Cignetti *et al.* 2005) to successfully co-infect primary MM cells. However in this case the fusogene strategy, which enables the monocistronic expression of multiple proteins as a single precursor (Gaken *et al.* 2000), was incorporated. This allows the transduction of target cells with multiple transgenes using only one lentiviral vector, providing a solution to the potential inefficiency and instability of gene transfer procedures and the problems linked to vector cytotoxicity (Gaken *et al.* 2000).

The results herein described show that the use of high titers of ultracentrifuge-concentrated virus, corresponding to a multiplicity of infection (MOI)¹ of 20, in combination with the infection enhancer polybrene represents the best strategy to obtain viable, stable and highly genetically-modified MM cell lines and CD138⁺ MM primary cells.

The genetically-modified MM cells were also sorted on the basis of their levels of CD80 expression to isolate a pure genetically-modified cell population for subsequent functional assays (see Chapter 3.3). These cells were also characterised in terms of viability and expression of CD28 and PD-L1, as their expression could potentially be affected by the transgene CD80 (Haile *et al.* 2011).

The results obtained demonstrate that lentiviral transduction and cell sorting have a minor impact on cell viability and that the expression of CD80 does not change the levels of expression of the markers CD28 and PD-L1.

¹ The multiplicity of infection (MOI) is defined as the ratio of virus genomes to infection target (in this case, MM cells) during infection (González-Jara *et al.* 2009).

3.2.2 Results

3.2.2.1 Construction and production of lentiviral vectors

The HIV-based lentiviral vectors LV.CD80, LV.IL-2 and LV.IL-2/CD80 (described in 2.2.1), were generated by transfection of the correspondent plasmid constructs in helper cells.

Before this step the plasmids, which were kindly provided by Dr. Chan, were expanded in Stbl3 *E. coli* cells and purified by Maxi Prep technique (as described in 2.2.2.1.5). The obtained DNA was tested for purity and concentration (as described in 2.2.2.1.3) and the identity of pLV.IL-2/CD80 was confirmed by diagnostic restriction digestion (as explained in 2.2.2.1.4 and illustrated in Figure 3-12). This last step was necessary as two versions of pLV.IL-2/CD80 were available in our department: one in which the enhancer WPRE was included to achieve high levels of transgene expression, and a second, which lacked this element in order to comply with the safety regulations required to be employed in phase I clinical studies. In order to compare the results with those previously obtained by Chan (Chan *et al.* 2005a), Ingram (Ingram *et al.* 2009) and Cignetti (Cignetti *et al.* 2005), the version that included WPRE was used in all the experiments.

The diagnostic restriction digestion carried out on pLV.IL-2/CD80 using the enzymes *EcoRI* and *SaI*, yielded four bands of expected sizes (7702bp, 992bp, 878bp, 603bp; Figure 3-12). An additional band of 1870bp was also noted. This represents an expected product when *EcoRI* is the only restriction enzyme present, and may suggest a higher efficiency of cut by the enzyme *EcoRI* compared to *SaI*. Nevertheless, this restriction pattern confirmed the presence of the correct construct of the human IL-2/CD80 cDNA in the plasmid.

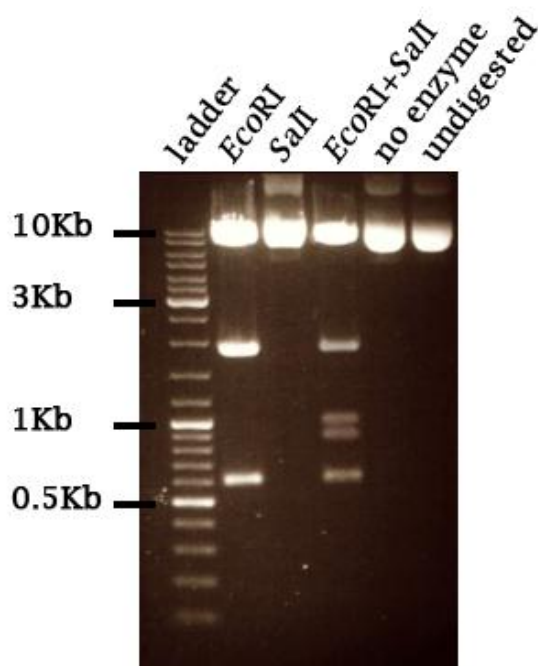


Figure 3-12 Gel electrophoresis pattern of plasmid restriction fragments. The products of the diagnostic restriction digestion (see 2.2.2.1.4) were resolved in agarose gel. The sizes of the fragments are indicated in kilobases (Kb).

After validating the plasmids, they were used to produce lentiviral vectors (as described in 2.2.2.2) and their concentration, defined as titer, was determined by infecting K562 cells and calculating the percentage of genetically-modified cells after 96 hours from the infection (Figure 3-13).

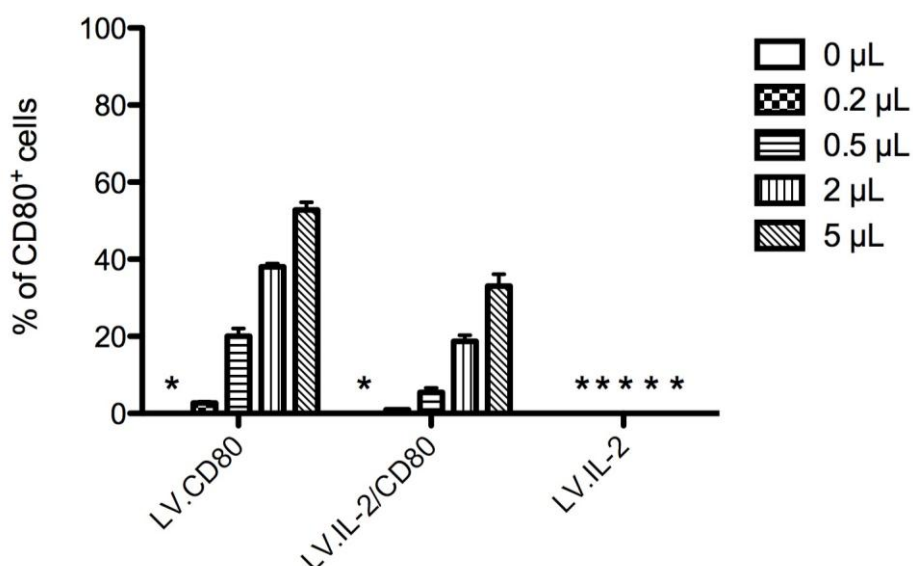


Figure 3-13 Lentiviral vectors titration in human erythroleukemic cell line K562. K562 cells were seeded at 5×10^5 cells/mL and infected as described in 2.2.2.3.2 using the indicated volumes of the appropriate vector. After 24 hours, cells were washed twice and cultured for 72 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

Virus titers were calculated according to the following formula:

$$\text{Titer } \left(\frac{\text{IU}}{\text{mL}} \right) = \text{No. of cells at the time of vector addition} \times \frac{\% \text{ of positive cells}}{100} \times \text{virus dilution factor}$$

Based on the flow cytometry results and the formula illustrated above, this protocol was able to concentrate 6.8×10^7 infective units (IU)/mL for LV.CD80 and 5.5×10^7 IU/mL for LV.IL-2/CD80 from a starting volume of 150 mL. Although no data is available on the titer before concentration, this result appears to be in line with that obtained by Chan and colleagues who, using the same technique, concentrated $5\text{--}7 \times 10^8$ IU of lentivirus vector starting from 450mL of 293T culture supernatant (Chan *et al.* 2005b). On this basis, it is possible to conclude that the vector concentration was successfully performed.

For cells infected with LV.IL-2, alternative techniques can be used to determine virus titer: estimation of genome copy number by quantitative PCR, p24 ELISA, which detects HIV-1 p24 antigen, or intracellular staining for IL-2. However, as the ultimate aim was not to evaluate the percentage of infected cells, but to compare IL-2 release in the culture medium from uninfected, LV.IL-2/CD80 and LV.IL-2 K562 cells, culture supernatants were tested by IL-2 ELISA (Figure 3-14).

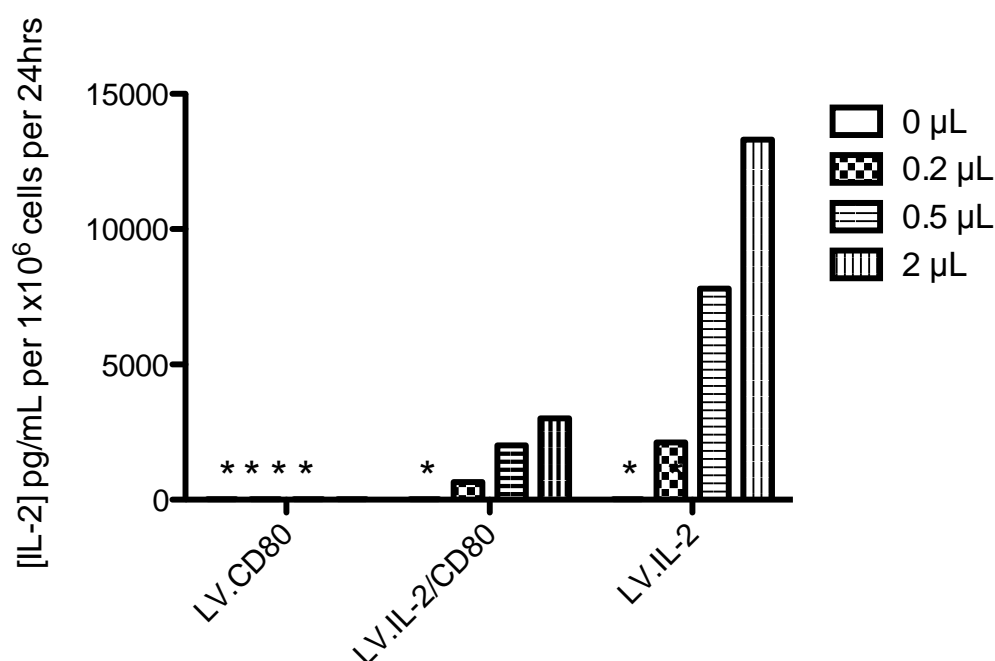


Figure 3-14 IL-2 production following infection with lentiviral vectors. K562 cells were seeded at 5×10^5 cells/mL and infected as described in 2.2.2.3.2 using the indicated volumes of the appropriate vector. After 24 hours, cells were washed twice, seeded at 1×10^6 cells/mL and cultured for another 24 hours. Culture supernatants were then harvested and tested for IL-2 concentration by ELISA. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

The results showed that infection with LV.IL-2/CD80 or LV.IL-2 induced IL-2 secretion, with equivalent volumes of LV.IL-2 resulting in significantly higher levels of IL-2 transgene expression: 13 ng/mL per 1×10^6 cells/24hours compared to 3 ng/mL per 1×10^6 cells/24hours obtained from LV.IL2/CD80 infected cells.

Taken together, these results confirmed the functional activity of the generated vectors and their ability to transduce target cells.

3.2.2.2 Optimisation of a protocol to transduce the MM cell line U266

The LVIL-2/CD80 vector was initially used to transduce U266 and K562 cells at a MOI of 5, representing a 5:1 ratio between the number of lentivirus infective units (IU) and the number of cells infected. For LV.IL-2 the same volume of lentiviral suspension calculated for LV.IL-2/CD80 was added.

The histograms reported in figure 3-15 show that U266 cells naturally express low levels of CD80 (about 9% CD80⁺ cells). This was taken into account when calculating virus titers and the efficiency of cell transduction. Infection with LV.IL-2/CD80 vector was able to transduce the MM cell line U266, although K562 cells appeared to be more readily infectable (77.3% CD80⁺ K562 cells compared to 30.8% of CD80⁺ U266 cells).

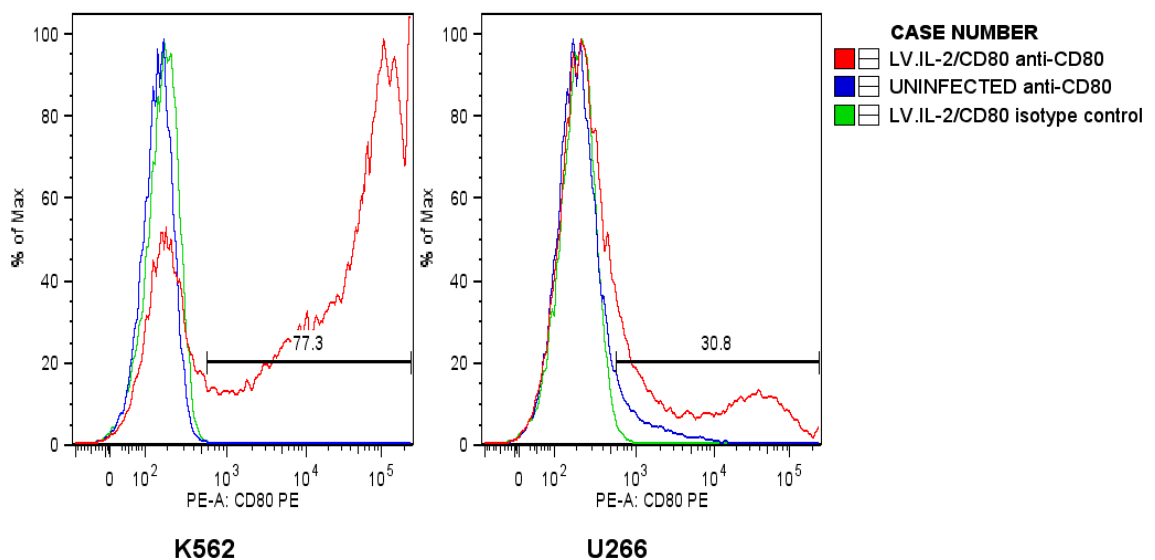


Figure 3-15 Comparison between K562 and U266 cell permissivity to LV.IL-2/CD80. K562 (left) and U266 cells (right) were infected as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 5 and cultured for 96 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls. Flow cytometry plots are representative of three independent experiments.

The staining of genetically-modified cells with a viability dye revealed that the transduction protocol had a cytotoxic effect on U266 cells (Figure 3-16). This phenomenon was not observed when using K562 cells. In fact, following infection with LV.IL-2/CD80 the percentage of dead U266 cells increased of a factor of 3 to reach 61.2% trypan blue positive cells, whereas the mean of percentage of dead K562 cells remained stable around 21.8%.

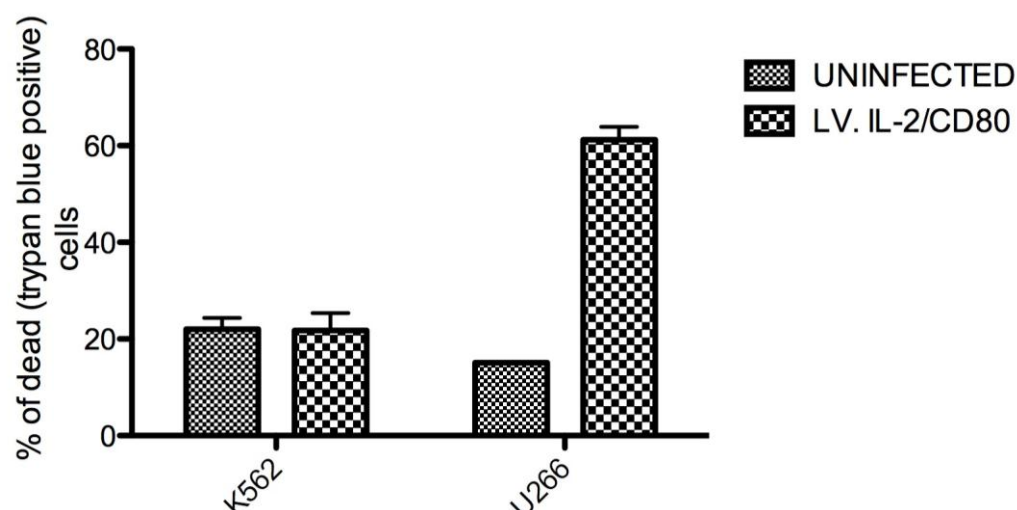


Figure 3-16 Effect of lentiviral transduction on cell viability. K562 and U266 cells were infected with LV.IL-2/CD80 as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 5 and cultured for 96 hours prior to analysis of cell viability by trypan blue test. Bars show the mean and standard deviation from replicate wells.

Subsequently, the cytotoxic effect of the virus, added alone or in combination with cationic polymers to the cell cultures, was investigated.

As shown in Figure 3-17, the presence or absence of the lentiviral vector alone does not account for the increase in cell death, whereas the presence, type and concentration of the infection enhancer seem to have a major impact. Of all the combinations screened, a low concentration of polybrene (4 μ g/mL) was able to reduce the infection-related cytotoxicity while not impacting the transduction efficiency (Figures 3-17 and 3-19).

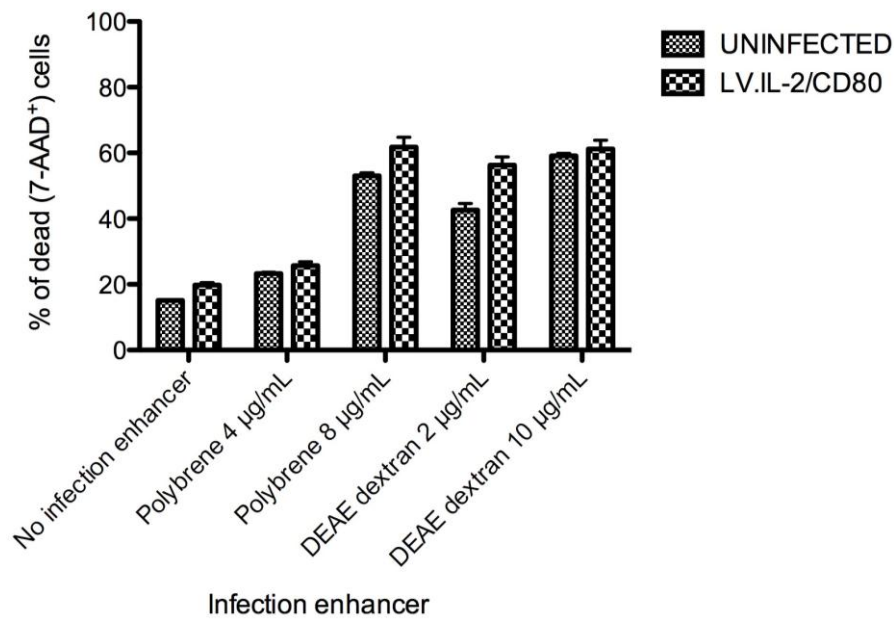


Figure 3-17 Impact of infection enhancers on U266 cell permissivity and viability. Human MM U266 cells were infected with LV.IL-2/CD80 as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 5 and in the presence of different concentration of infection enhancers and cultured for 96 hours prior to analysis of cell viability by flow cytometry (7-AAD staining). Bars show the mean and standard deviation from replicate wells.

The decreased cytotoxic effect induced by this concentration of polybrene was also confirmed using the MTT assay (Figure 3-18) as an alternative method to detect cell viability and proliferation.

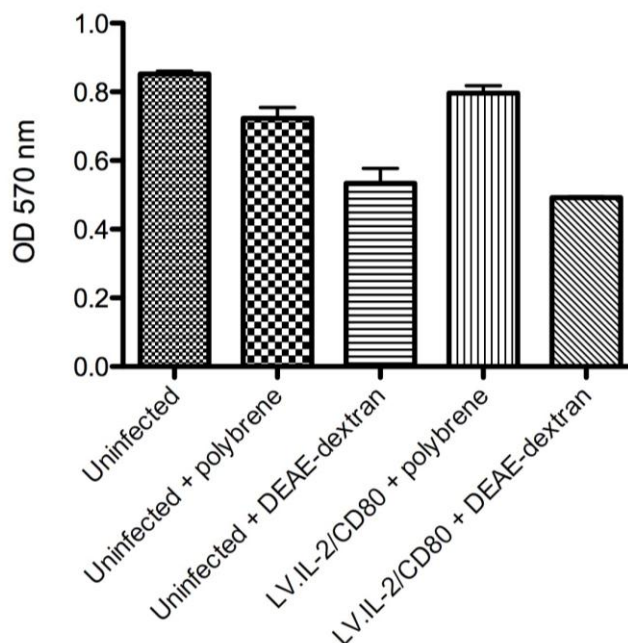


Figure 3-18 Impact of infection enhancers on U266 cell proliferation and viability. Human MM U266 cells were infected with LV.IL-2/CD80 as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 5 in the presence of polybrene (4 µg/mL) or DEAE-dextran (2 µg/mL) and cultured for 96 hours before evaluation of cell proliferation and viability by MTT assay. Bars represent the mean and standard deviation of 570nm absorbance readings, which positively correlates with cell viability, obtained from replicate wells.

However, despite the improvement in cell viability, it was not possible to reach levels of transduction similar to those of K562 cells (Figure 3-19).

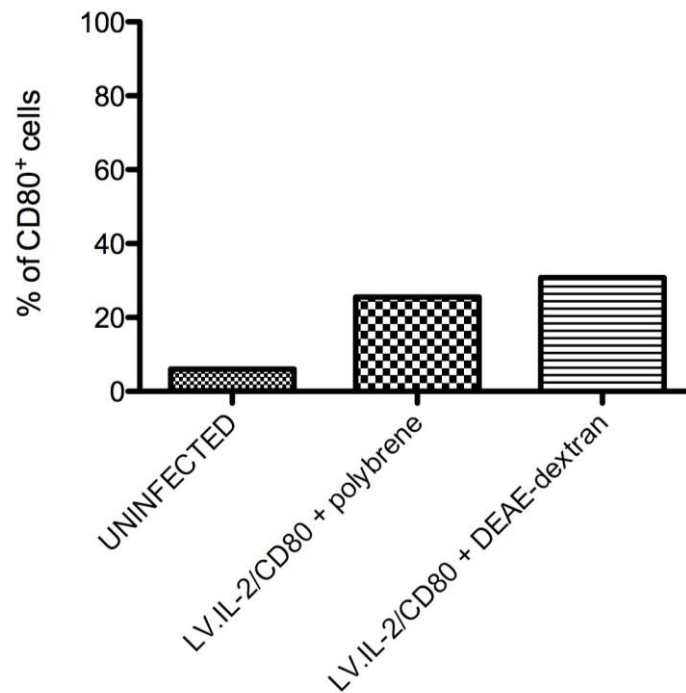


Figure 3-19 Effect of different infection enhancers on U266 cell transduction. U266 cells were infected as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 5 in absence or presence of the infection enhancer polybrene (4 μ g/mL) or DEAE-dextran (2 μ g/mL). Cells were cultured for 96 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. Bars represent the mean and standard deviation of the percentage of CD80⁺ cells obtained from replicate wells.

Although Chan and colleagues (unpublished data) presented evidence supporting the hypothesis that even a small population of genetically-modified AML cells (as low as 20% of CD80⁺ cells) is able to trigger T cell expansion and activation, there were still concerns that, in this MM system, the levels of activation induced by genetically-modified cells may strongly vary according to the expression of the transgenes. On the basis of these considerations and with the aim to compare this whole cancer cell vaccine strategy with other strategies expressing high levels of transgenes (Tarte *et al.* 1999; Wen *et al.* 2001a; Cignetti *et al.* 2005; Ren *et al.* 2006; Lu *et al.* 2007) different methods to achieve higher and comparable transduction levels were investigated.

As shown in figure 3-15, permissivity to viral infection appears to be cell-line specific and could depend, amongst other factors, on the virus-induced cytotoxicity and

the interaction between the virus and the cell surface (Chan *et al.* 2005b). Therefore, alternative strategies to maximise the interaction of viral particles with the target cell surface were evaluated.

Biotin-labelled lentiviral vectors were generated through the use of the BL-15 293T-derived human packaging cell line (Nesbeth *et al.* 2006) (see 2.2.2.2.2). The lentivirus concentration was then increased 56 fold (concentrating 56 mL of lentiviral suspension to 1 mL of lentiviral-PMP suspension) and virus titers were determined on the basis of the detection of CD80 on the surface of K562 and U266 cells (Figure 3-20) and by using the formula reported in paragraph 3.2.2.1.

This analysis showed that the starting titer was concentrated 160 times for K652 (from 1.75×10^6 IU/mL to 2.8×10^8 IU/mL) and 50 times for U266 (from 7.5×10^5 to 5×10^7 IU/mL).

Figure 3-20 also shows that the PMP-conjugated LV.IL-2/CD80 vector was able to effectively transduce the myeloma cell line U266, achieving 58% of transgene expression, when using 100 μ L of PMP-LV.IL-2/CD80 (corresponding to an MOI of 48) and 43% when using 10 μ L of PMP-LV.IL-2/CD80 (corresponding to an MOI of 4.8). The results obtained, whilst confirming the low permissivity of U266 cell, also suggest that PMP-conjugated virus may improve viral presentation to target cells. In fact, when using comparable MOI of virus (4.8 versus 5) the use of PMP-LV.IL2/CD80 lead to a higher percentage of genetically-modified cells when compared with LV.IL2/CD80 (43% compared to 25%).

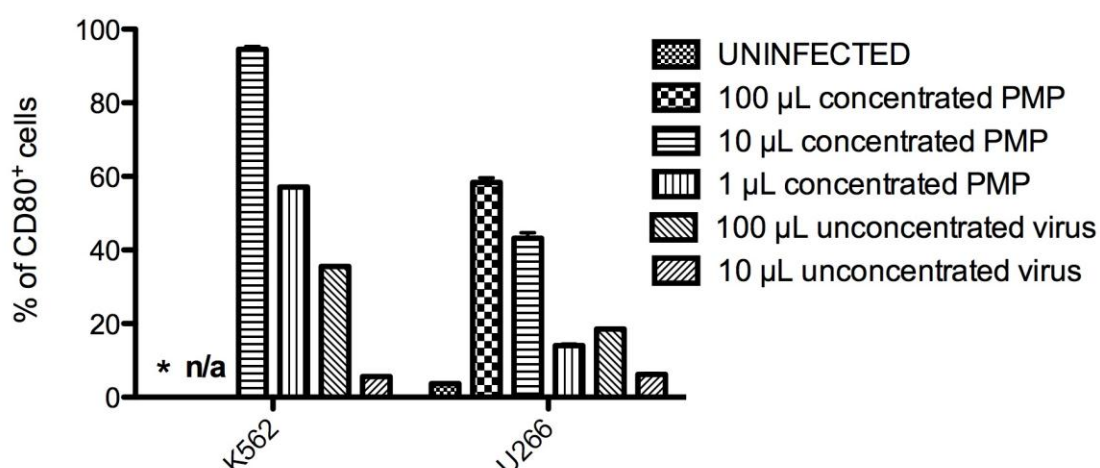


Figure 3-20 Comparison between K562 and U266 cell permissivity to LV.IL-2/CD80-conjugated with PMP and evaluation of lentiviral concentration efficiency.

K562 and U266 cells were seeded at 5×10^5 cells/mL and incubated in the presence of 4 µg/mL of polybrene and infected as described in 2.2.2.3.1 using the indicated volume of PMP-conjugated LV.IL-2/CD80 vector. Cells were then washed twice and cultured for 72 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. * indicates results below the detection limit of the assay while n/a stands for “not available” as that particular condition was not performed. Bars represent the mean and standard deviation of the percentage of CD80⁺ cells obtained from replicate wells.

Interestingly, increasing volumes of PMP-conjugated virus did not result in a comparable increase in the percentage of transduced U266 as happened with K562 (Figure 3-20). The high infection-related cytotoxicity to U266 cells (Figure 3-21) may explain this phenomenon and the reason why the percentage of genetically-modified U266 cells reaches a plateau at approximately 60% of CD80 positive cells.

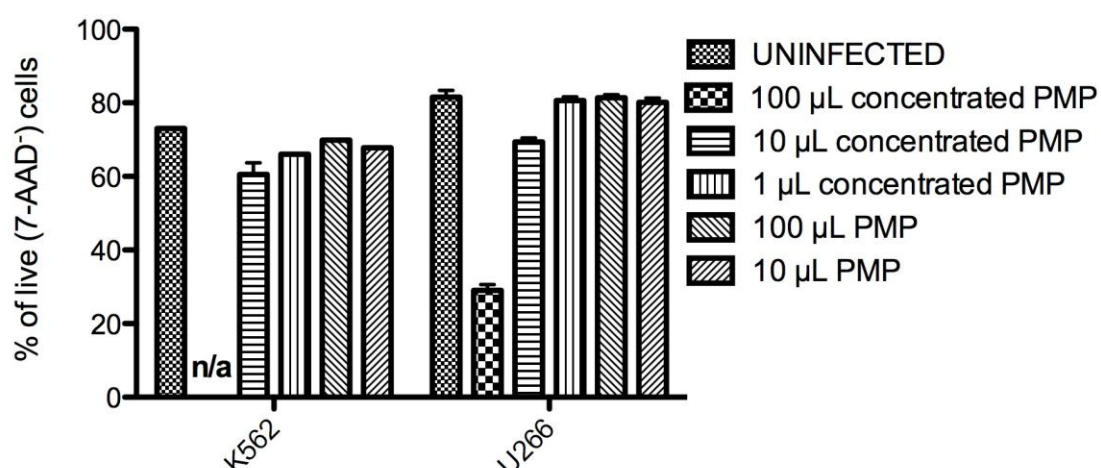


Figure 3-21 Effect of lentiviral transduction with PMP-conjugated virus on cell viability. K562 and U266 human cells were infected as described in 2.2.2.3.1 and Figure 3-20. PMPs were then removed through the use of a magnet and the cells were washed twice and cultured for 72 hours prior to analysis of cell viability by flow cytometry (7-AAD staining). n/a stands for “not available” as that particular condition was not performed. Bars represent the mean and standard deviation of the percentage of live (7-AAD⁻) cells obtained from replicate wells.

Since the percentage of viable U266 cells infected with 10 µL of PMP-conjugated lentivirus was similar to that of uninfected cells (81.6% 7-AAD⁻ cells for U266 cells compared to 69.4% 7-AAD⁻ cells for LV.IL-2/CD80 U266 cells), an alternative transduction strategy was tested with the aim of achieving a better transgene expression while using a smaller volume of PMP.

The polycation polyethylenimine (PEI) was combined with PMP-conjugated LV.IL-2/CD80 to maximise virus interaction with target cells. Additionally, target cells were biotinylated to increase their interaction with PMP-lentiviral particles (Figure 3-22).

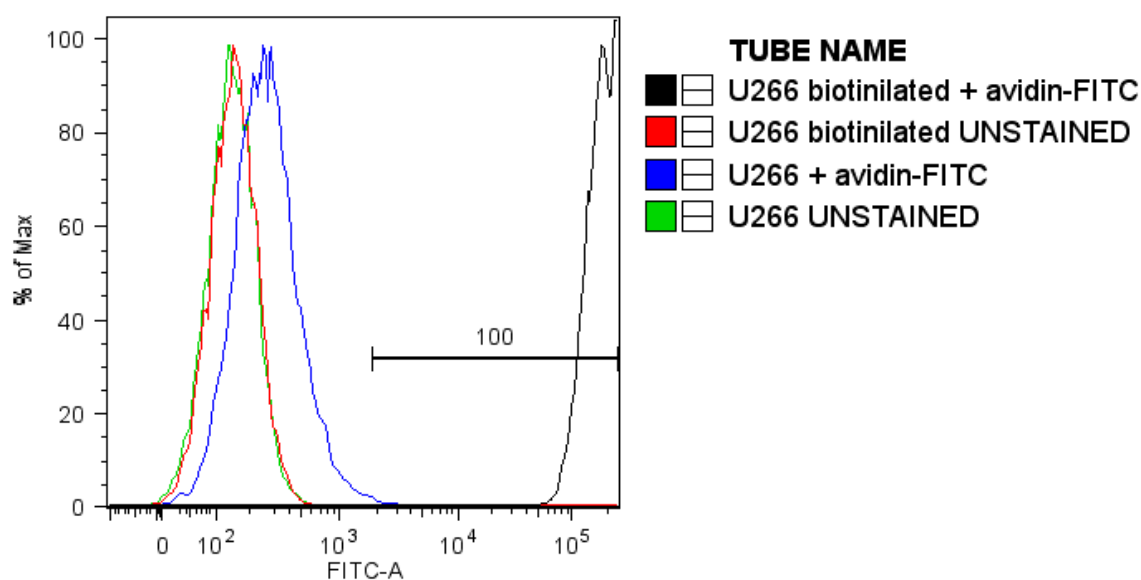


Figure 3-22 Biotinilation of U266 cells.

U266 cells were resuspended in DPBS and incubated with 3mM of biotin for 30 minutes, washed twice and resuspended in fresh RPMI complete medium. The presence of biotin linked to the cell surface was detected through staining with FITC-labelled avidin. All labelling was performed with unstained controls for each sample. Histograms are representative of replicate wells.

Figure 3-22 confirms the successful biotinitation of U266 cells. The shift in the peak of non-biotinitated avidin-labelled U266 cells is likely to be due to the non-specific binding of avidin to the cells.

Next, the advantage of this strategy was evaluated by calculating the fold increase in the percentage of genetically-modified U266 cells over untreated cells, following the described treatments (Figure 3-23). The results show that none of the strategies considered was able to increment the transduction efficiency.

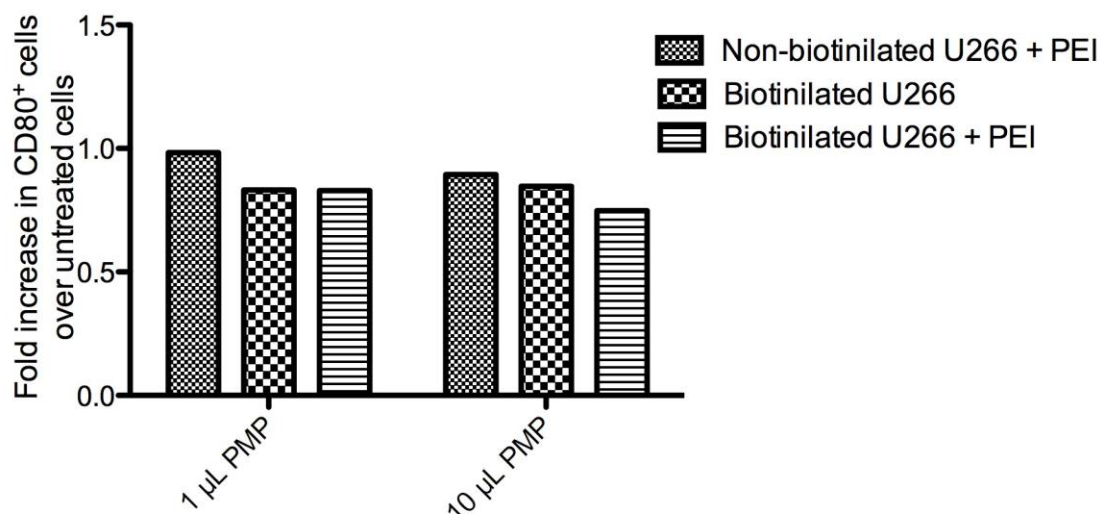


Figure 3-23 Effect of PEI treatment and target cells biotinylation on the percentage of genetically-modified cells following infection with LV.IL-2/CD80.

U266 and biotinilated U266 cells were resuspended at a concentration of 5×10^5 cells/mL in RPMI complete medium. Cells then received an appropriate amount of PMP-conjugated LV.IL-2/CD80 in the presence of $4 \mu\text{g/mL}$ of polybrene. When indicated, PEI was added to the cultures. Results are expressed as fold increase in the percentage of CD80⁺ cells over untreated (non-biotinilated cells infected with the correspondent amount of PMP-LV.IL-2/CD80 in the absence of PEI). Bars represent the mean obtained from replicate wells.

Nevertheless, the results obtained using 100 µL of PMP-lentiviral particles (which correspond to an MOI of 48; Figure 3-19) suggested that the use of higher MOI might increase the percentage of infected U266 cells. Considering the high cytotoxicity levels induced by the use of large quantities of lentiviral-PMP suspension, a new batch of lentiviral vector was generated and concentrated by ultracentrifugation. Specifically, a 240 fold concentration was performed, obtaining 1mL of lentivirus suspension from a starting volume of culture supernatant of 240 mL.

The titration results showed that this protocol was able to produce functional viruses characterised by a titer of 3.06×10^8 IU/mL for LV.IL-2/CD80 and 3.39×10^8 IU/mL for LV.CD80, when K562 are used as target cells, and 5.58×10^7 IU/mL for LV.IL-2/CD80 and 1.47×10^8 IU/mL for LV.CD80, when U266 cells are used as target cells (Figure 3-24).

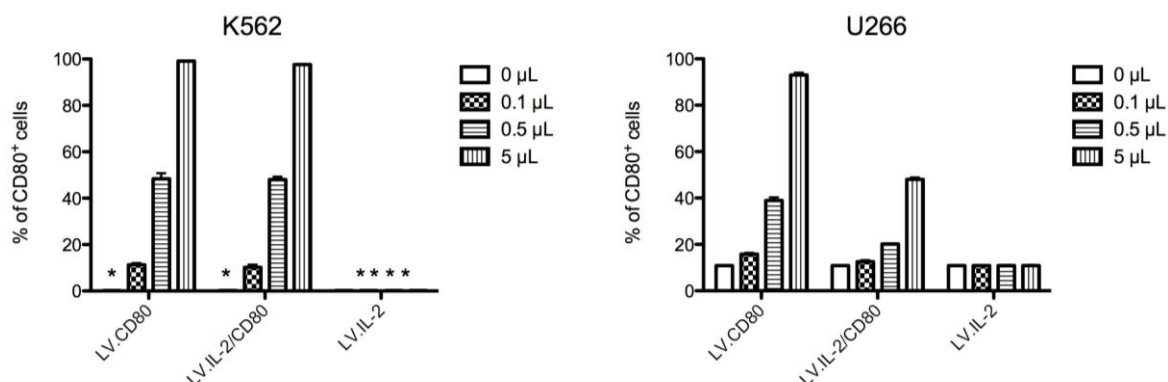


Figure 3-24 Lentiviral vector titration in K562 and U266 cell lines. K562 (left) and U266 cells (right) were infected as described in 2.2.2.3.2 and Figure 3-13 using the indicated volume of vector resuspension. Cells were washed twice and cultured for 72 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

U266 cells were then infected with LV.IL-2/CD80 at an MOI of 20 in the presence of the infection enhancer polybrene. The histograms in Figure 3-25 show that this lentiviral vector successfully transduces U266 cells resulting in high levels of transgene expression.

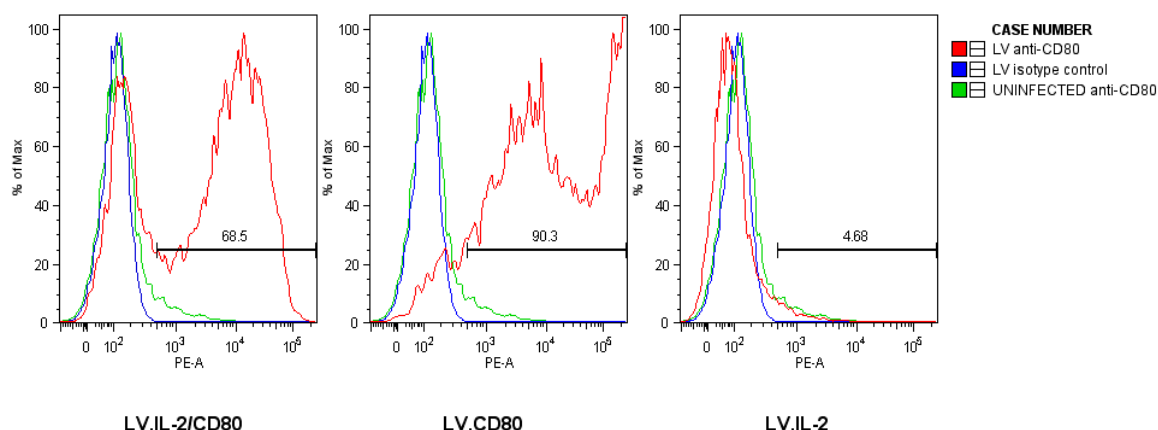


Figure 3-25 CD80 expression levels following infection with lentiviral vectors. U266 cells were infected as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 20. Cells were cultured for 96 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. The flow cytometry plots are representative of three replicate wells.

Although approximately 90% of U266 cells were successfully modified when using LV.CD80, it was possible to distinguish two different populations of genetically modified cells on the basis of CD80 expression levels (Figure 3-25).

Follow up studies showed that the different expression levels of CD80 appear to be cell specific, as infected RPMI8226 cells (3-27) present a homogeneous and stable phenotype in terms of transgene expression. This phenomenon is also not transient, as CD80 levels and MFI were stable for 30 days in both the CD80^{high} and CD80^{dim} cell populations (Figure 3-26).

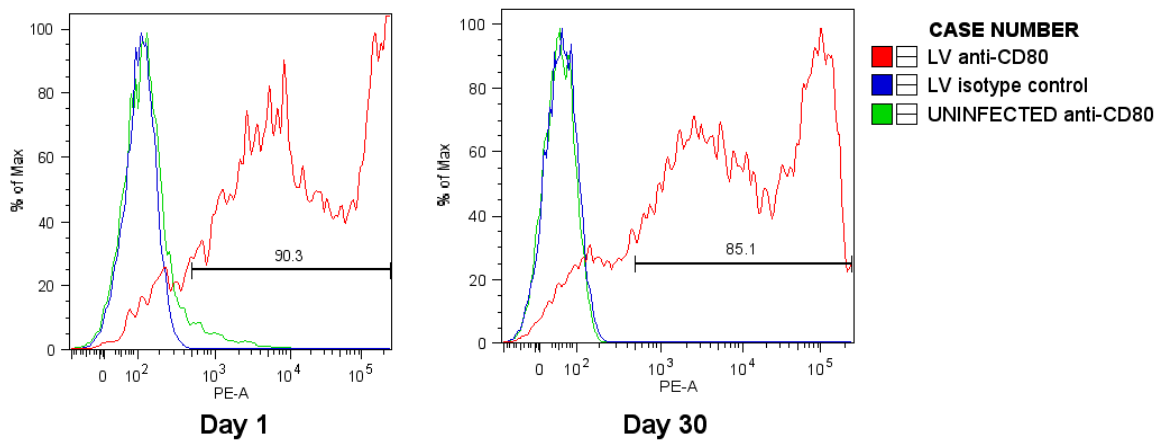


Figure 3-26 Stability of the CD80^{high} and CD80^{dim} peaks over time. LV.CD80 U266 cells were analysed for CD80 expression by flow cytometry 96 hours post infection and 30 days post-infection. All labelling was performed with matched isotype and unstained controls for each sample. The flow cytometry plots are representative of three replicate wells.

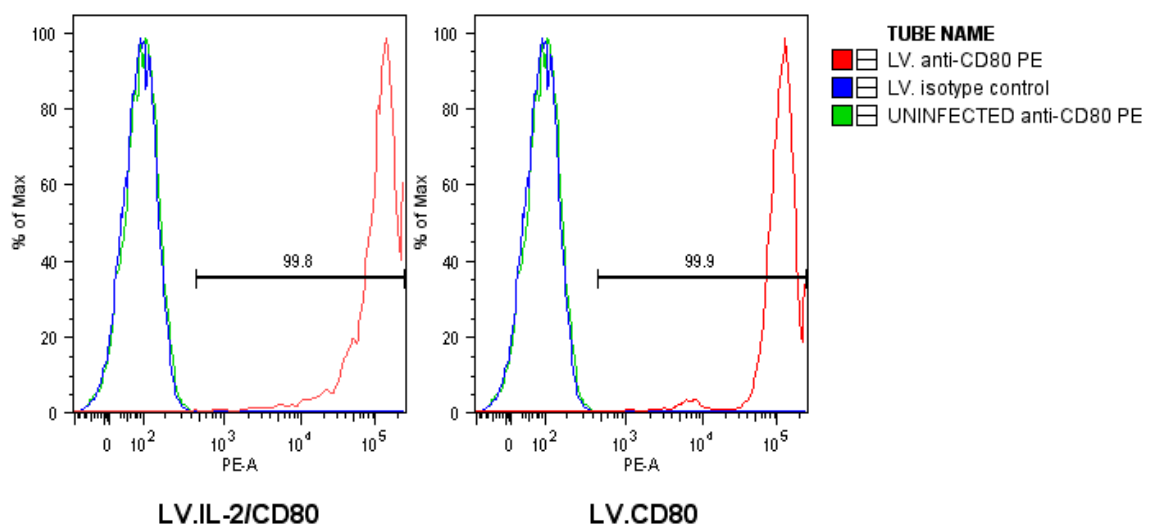


Figure 3-27 CD80 expression levels in RPMI8226 cells following infection with lentiviral vectors. RPMI8226 cells were infected as described in 2.2.2.3.2 and Figure 3-13 at an MOI of 20. Cells were cultured for 96 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. The flow cytometry plots are representative of three replicate wells.

To exclude that the CD80 expression profile observed in modified U266 (Figure 3-25 and 3-26) was caused by the use of a heterogeneous cell line, the U266 cells in use were phenotypically examined by flow cytometer. The obtained data suggest that the cell line in use is homogeneous in terms of granularity and size (Figure 3-28). Additionally, CD80^{high} and CD80^{dim} cells cannot be segregated on the basis of their FSC and SSC profiles, which could have indicated the presence of two different populations characterised by a different permissivity to lentiviral infection (Figure 3-28).

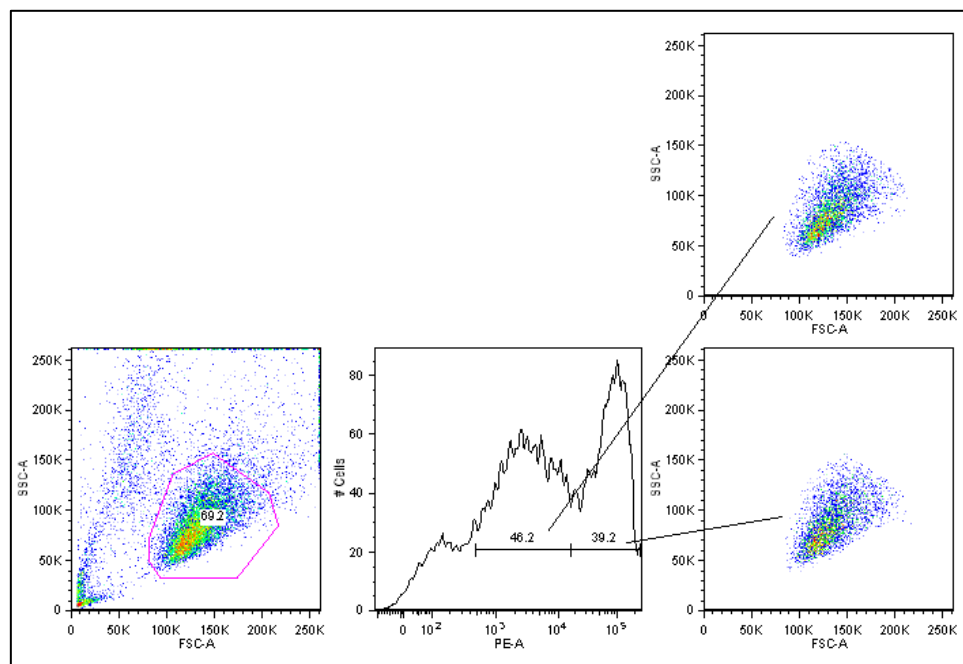


Figure 3-28 FSC and SSC characterisation of CD80^{high} and CD80^{dim} peaks. LV.CD80 U266 cells were analysed for CD80 expression (center) 96 hours post infection by flow cytometry using an anti-CD80PE antibody. The CD80^{high} (top right panel) and CD80^{dim} (bottom right panel) positive cells were then characterised for their FSC vs SSC profiles and compared with the FSC vs SSC profile of the whole cell population (left). Flow cytometry plots are representative of three replicate wells.

Secondly, its identity and purity were confirmed by the homogeneous expression of known extracellular markers, such as CD138 and CD28 (see paragraph 2.1.2.2).

3.2.2.3 Generation of genetically-modified U266 cells for functional assays

As subsequent assays required the genetically-modified MM cell to express comparable levels of the transgene(s), transduced cells were sorted on the basis of their CD80 expression levels. When choosing whether to sort the high or the dim population within LV.CD80 U266 cells it was considered that, in order to be able to compare the stimulatory ability of CD80 alone or in the presence of IL-2, the percentage and MFI of LV.CD80 U266 cells should be equivalent or greater than those of LV.IL-2/CD80 cells. Consequently, the CD80^{high} population was selected, as the MFI of the CD80^{dim} population of LV.CD80 U266 was lower compared to that of CD80⁺ LV.IL-2/CD80 U266 cells.

The histograms shown in figure 3-29 demonstrate the successful isolation of CD80^{high} positive cells.

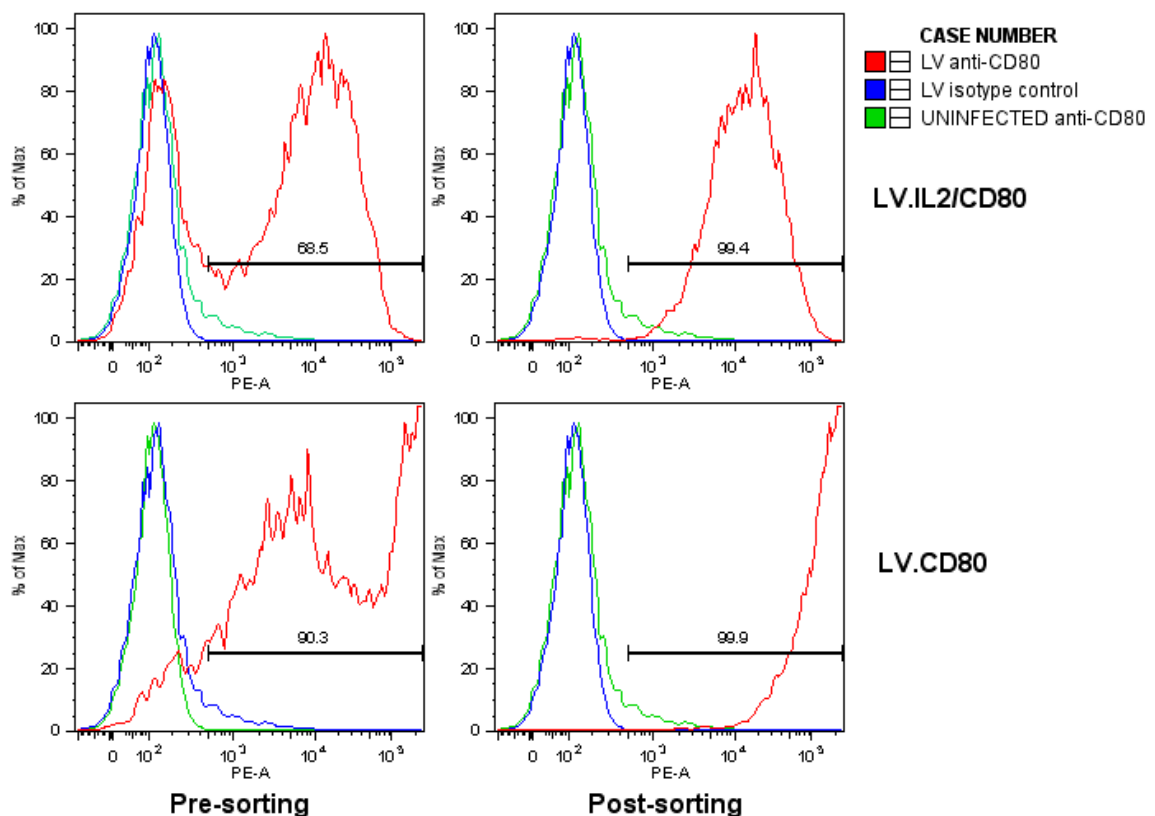


Figure 3-29 CD80 expression levels pre and post-fluorescence-activated cell sorting. Previously modified cells, as described in 2.2.2.3.2 and Figure 3-13, were expanded and CD80 positive cells were isolated using fluorescence activated cell sorting. The histograms show the levels of CD80 expression in the pre and post-sorted cells. All labelling was performed with matched isotype and unstained controls for each sample.

Sorted cells were subsequently tested for IL-2 production. Figure 3-30 shows that LV.IL-2 U266 cells produce considerably higher quantities of IL-2 when compared to LV.IL-2/CD80 U266 cells.

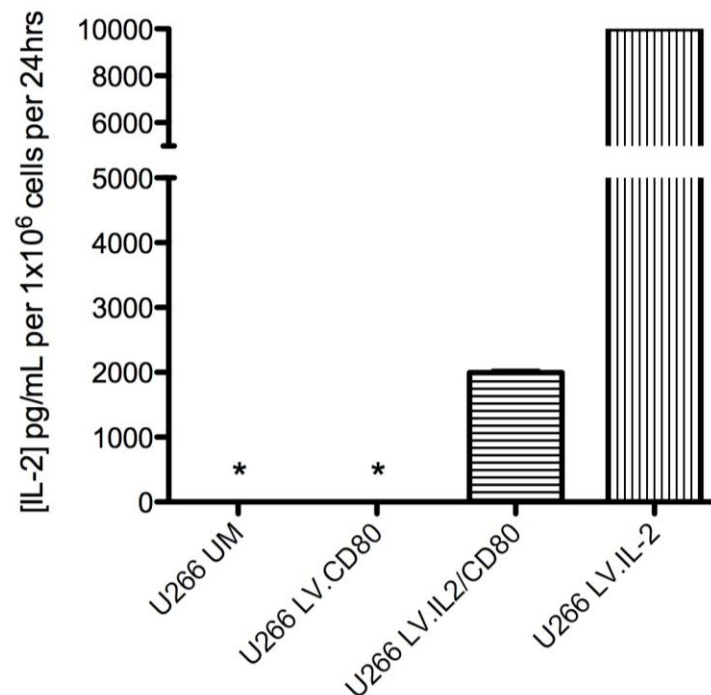


Figure 3-30 IL-2 production following infection with lentiviral vectors. LV.IL-2 U266 and sorted LV.IL-2/CD80 and LV.CD80 U266 cells were seeded at 1×10^6 cells/mL and cultured for 24 hours. Culture supernatants were then harvested and tested for IL-2 concentration by ELISA. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

In order to be able to compare the stimulatory ability of LV.IL-2/CD80 with IL-2 genetically-modified U266 cells, serial dilutions of LV.IL-2 infected cells with unmodified U266 cells were performed to achieve IL-2 levels in the culture supernatant comparable to those secreted by LV.IL-2/CD80 U266 cells.

The results reported in figure 3-31 demonstrate that a 1:10 dilution of LV.IL-2 modified U266 cells with unmodified U266 cells is able to reduce IL-2 secretion to levels comparable to those of LV.IL-2/CD80 modified cells.

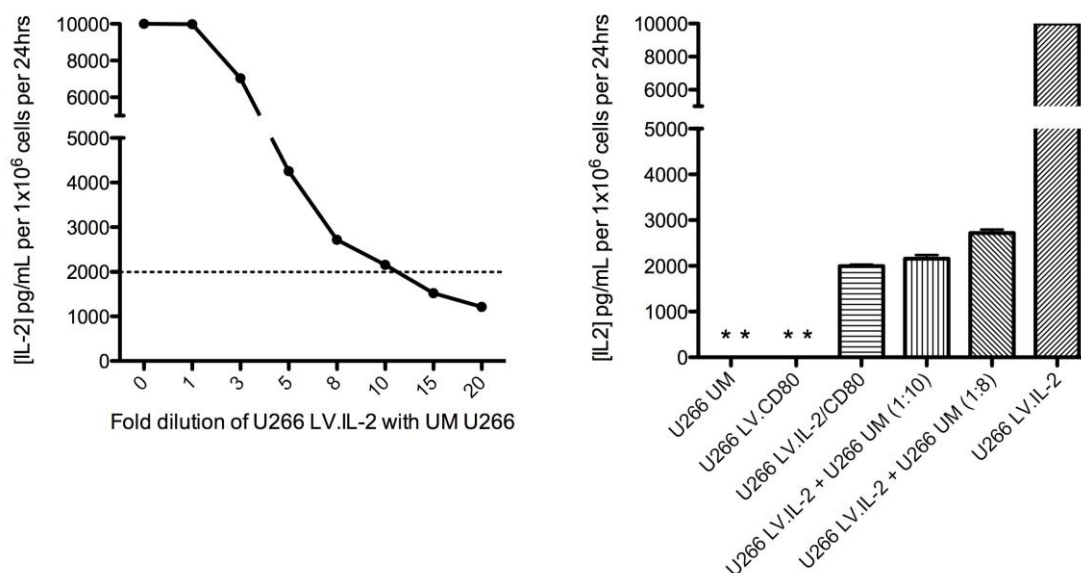


Figure 3-31 Comparison and normalisation of IL-2 secretion levels for LV.IL-2 and LV.IL-2/CD80 modified U266 cells.

Unmodified (UM), LV.CD80, LV.IL-2 and LV.IL2/CD80 U266 cells were seeded at 1×10^6 cells/mL. U266 LV.IL-2 cells were also mixed with U266 UM cells at different ratios. After 24 hours supernatants were harvested and tested for IL-2 concentration by ELISA. (Left) IL-2 release by LV.IL-2 U266 cells mixed with UM U266 cells at the indicated ratios. (Right) IL-2 release by UM, LV.CD80, LV.IL-2/CD80, LV.IL-2 U266 cells and LV.IL-2 U266 mixed with U266 UM cells at different ratios. * indicates results below the detection limit of the instrument. Bars show the mean and standard deviation from three independent experiments.

3.2.2.4 Characterisation of genetically-modified U266 cells

Next, characteristics of uninfected and infected U266 MM cells were compared using a panel of different parameters.

Firstly, the effect of lentiviral transduction on cell viability was evaluated through 7-AAD staining. Figure 3-32 shows that lentiviral transduction has a minor effect on cell viability.

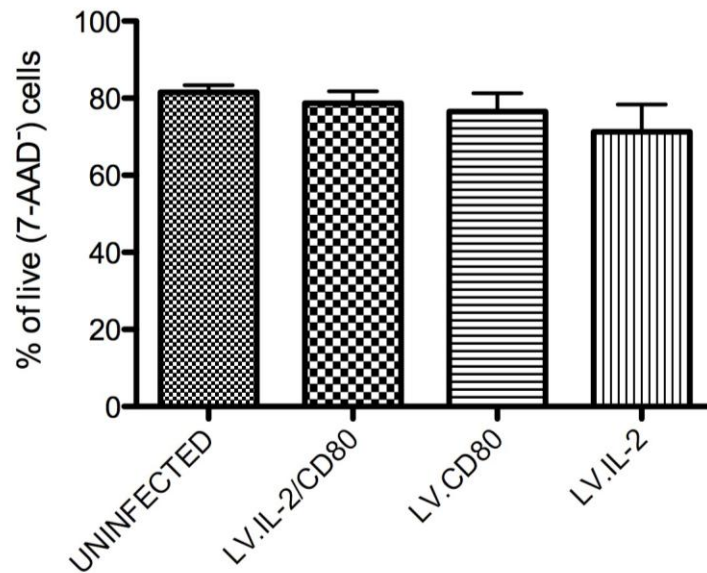


Figure 3-32 Effect of lentiviral transduction on cell viability. Uninfected and infected U266 cells were cultured in complete RPMI medium and analysed for cell viability by 7-AAD staining. Bars represent the mean and standard deviation of three independent experiments.

The stability of CD80 and IL-2 expression over time was also examined in genetically-modified U266 cells. Figure 3-33 shows a comparison between the CD80 flow cytometry profiles of LV.IL-2/CD80 and LV.CD80 U266 cells at day 1 and day 30 after cell sorting. Although there appears to be a decrease in the intensity of the CD80 positive peak, the controls indicate that this decrease is an artifact most likely caused by voltage fluctuations of the flow cytometer between uses rather than a loss of CD80 expression.

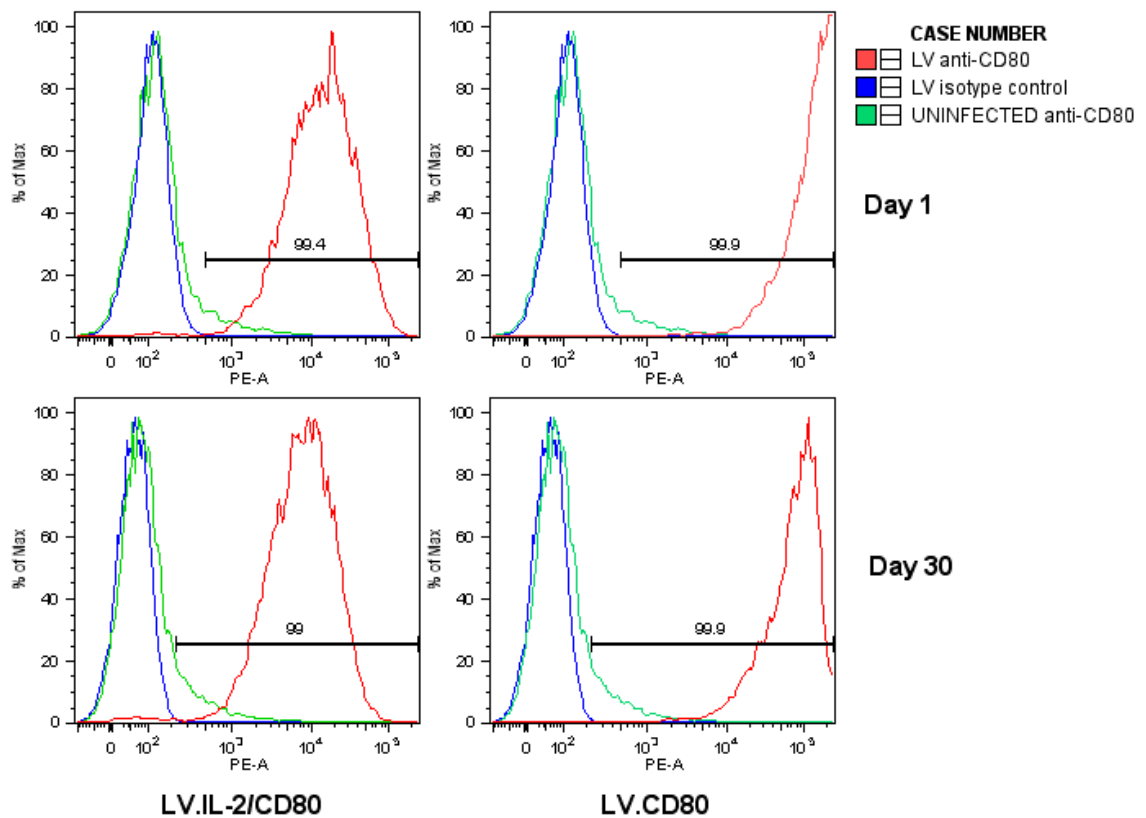


Figure 3-33 Stability of CD80 expression levels after fluorescence-activated cell sorting of CD80 positive cells.

Previously modified cells (see Figure 3-25) were cultured and expanded in complete RPMI medium for one day or 30 days. Cells were then washed twice and stained with anti-CD80PE. All labelling was performed with matched isotype and unstained controls for each sample. The histograms are representative of three independent experiments.

These studies demonstrated the stability of CD80 expression for at least 30 days of continuous *in vitro* culture both in LV.CD80 and LV.IL-2/CD80 modified U266 cells.

Likewise, Figure 3-34 shows that the levels of IL-2 production from LV.IL-2 and LV.IL-2/CD80 modified cells remain stable the same period after transduction. Only a slight increase in IL-2 secretion was noted after 30 days (about 1.17 fold) for both LV.IL-2/CD80 and LV.IL-2 modified cells.

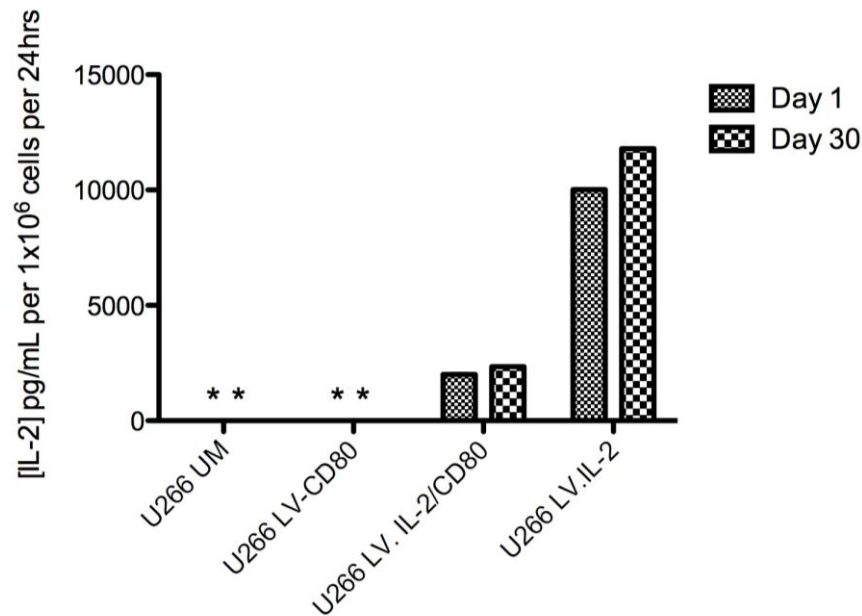


Figure 3-34 IL-2 production levels from uninfected or infected U266 cells on day 1 and day 30 post-sorting.

Previously modified cells (see Figure 3-25) were cultured and expanded in complete RPMI medium for one day or 30 days. On day 0 and on day 29 cells were seeded at 1×10^6 cells/mL and cultured for 24 hours. Culture supernatant were then harvested and tested for IL-2 concentration by ELISA. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

Based on the stability studies, modified and un-modified U266 cells were used for only up to 30 days of continuous culture. After this time, frozen aliquots of the original batch of cells were thawed and used for subsequent studies.

In a similar melanoma transduced system, Haile and colleagues showed that CD80 expression blocks the cell surface expression of the immunosuppressive receptor PD-L1 (Haile *et al.* 2011). To test this hypothesis on our genetically-modified cells, PD-L1 upregulation on the surface of U266 cells following culture with IFN- γ was evaluated. Healthy donor PBMCs were used as a comparison.

However, as shown in figure 3-35, the MM cell line U266 did not express a detectable level of PD-L1, even in the presence of IFN- γ levels able to trigger a profound up-regulation of PD-L1, from 1.55% to 84% PDL-1⁺ cells, on healthy donor PBMCs. Consequently, the downregulation of PD-L1 on CD80-modified cells was not further investigated.

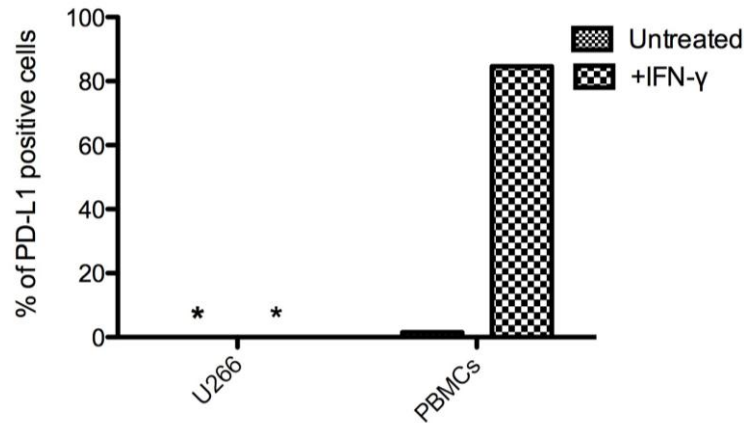


Figure 3-35 Evaluation of PD-L1 expression on U266 MM cell line and healthy donor PBMCs before and after treatment with IFN- γ . U266 cells and PBMCs were cultured in RPMI complete medium. When specified, 500 IU/mL of IFN- γ was added to the culture medium and cells were incubated for 24 hours. Cells were washed twice and analysed for PD-L1 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. * indicates results below the detection limit of the assay. The bars are representative of three independent experiments.

Finally, the expression of CD28, one of the main receptors for CD80 (Azuma *et al.* 1992), on the surface of the infected and uninfected MM cells was evaluated. The results demonstrated that the transduction of U266 cells with CD80 does not modify either the proportion of CD28 positive cells or the intensity of CD28 expression (Figure 3-36).

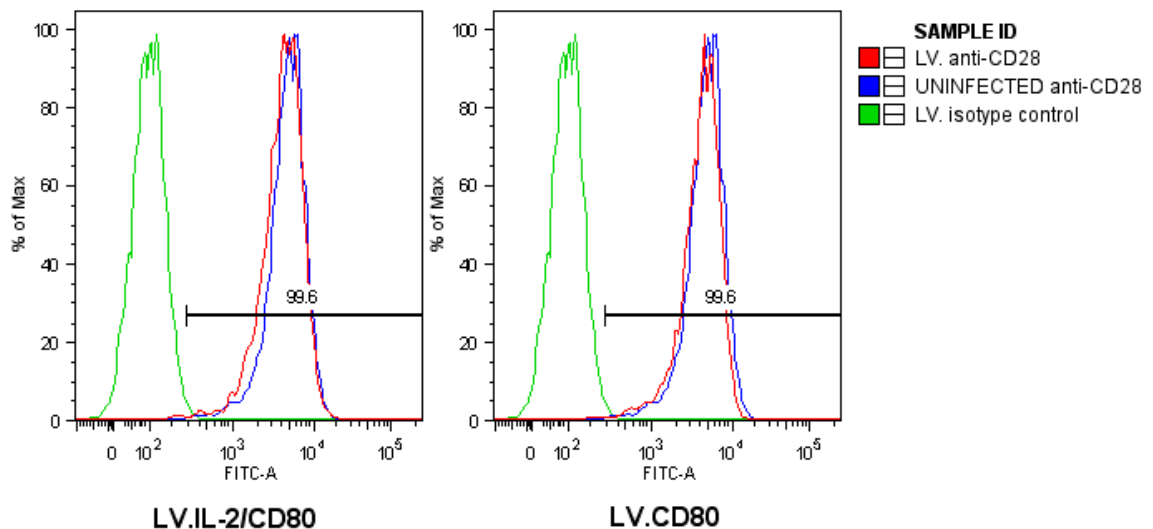


Figure 3-36 CD28 expression levels on U266 cells following transduction with LV.CD80 or LV.IL-2/CD80.

Uninfected and previously modified U266 cells (see Figure 3-25) were cultured and expanded in complete medium for 72 hours. Cells were then washed twice and analysed for CD28 expression by flow cytometric analysis. All labelling was performed with matched isotype and unstained controls for each sample. The histograms are representative of three independent experiments.

3.2.2.5 Genetic modification of CD138⁺ MM plasma cells

Purified CD138⁺ MM cells from one MM patient (see paragraph 2.1.4) were infected with LV.IL-2/CD80 in order to show the ability of the vector to transduce primary MM cells as well as MM cell lines. This was achieved using a protocol previously optimised in Cignetti (Cignetti *et al.* 2005), which utilises an MOI comparable to that used in the previous assays with U266 cells.

Analysis of CD80 expression confirmed that primary MM cells can be efficiently genetically-modified with the lentiviral vector LV.IL-2/CD80 (Figure 3-37).

The use of the infection enhancer polybrene was able to increase the MFI of CD80⁺ cells of about 5 fold (from 2593 to 10019), which suggest the integration of multiple vectors into once single cell, in these conditions.

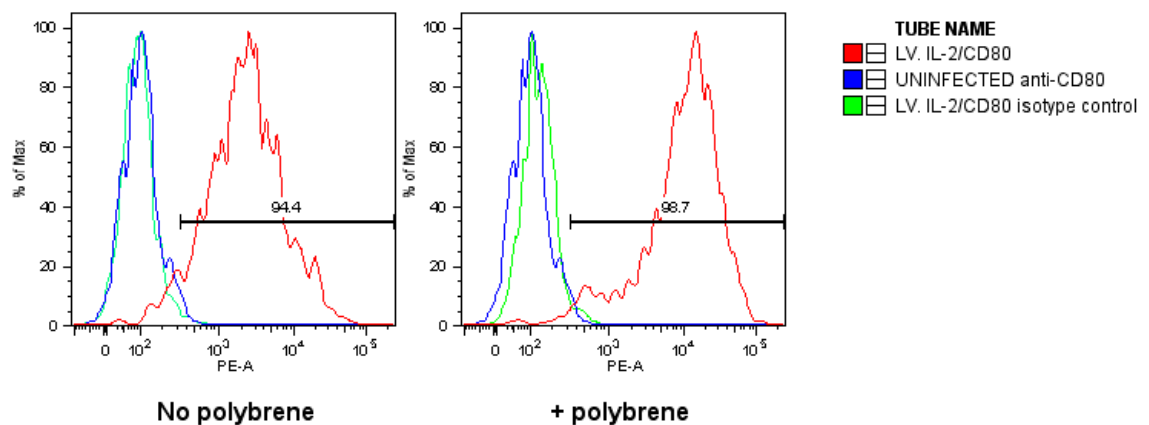


Figure 3-37 CD80 expression levels following infection of MM primary cells with the lentiviral vector IL-2/CD80.

CD138⁺ primary MM cells were infected as described in 2.2.2.3.2 at MOI of 20, under standard culture conditions. Cells were then washed twice and cultured for 72 hours prior to analysis of CD80 expression by flow cytometry. All labelling was performed with matched isotype and unstained controls for each sample. The flow cytometry plots are representative of three replicate wells.

The transduction of primary CD138⁺ MM cells with LV.IL-2/CD80 resulted in the production of 1.5 ng/mL of IL-2 in 24 hours by 1x10⁶ cells (Figure 3-38).

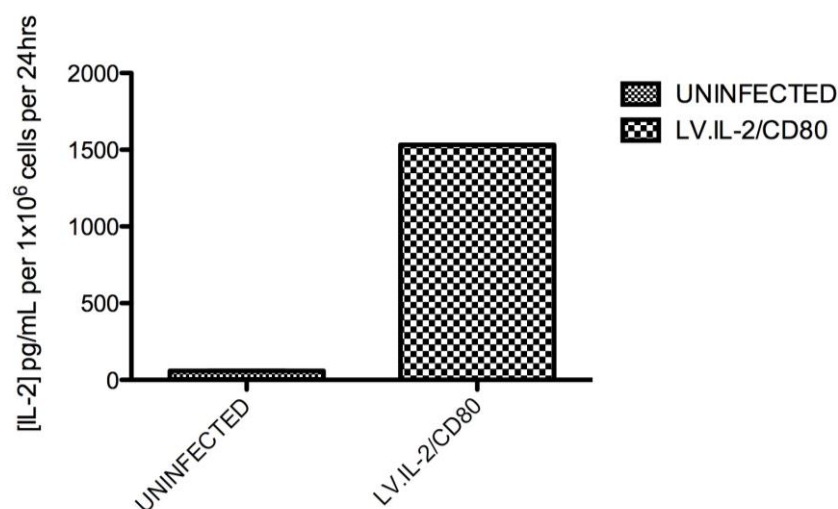


Figure 3-38 IL-2 production by LV.IL-2/CD80 CD138⁺ primary MM cells following infection with lentiviral vectors. LV.IL-2/CD80 CD138⁺ cells were seeded at 1x10⁶ cells/mL and cultured for 24 hours. Culture supernatant were then harvested and tested for IL-2 concentration by ELISA. * indicates results below the detection limit of the assay. Bars represent the mean and standard deviation from replicate wells.

Viability staining with 7-AAD showed that the lentiviral infection did not cause any significant change in MM cell viability (Figure 3-39).

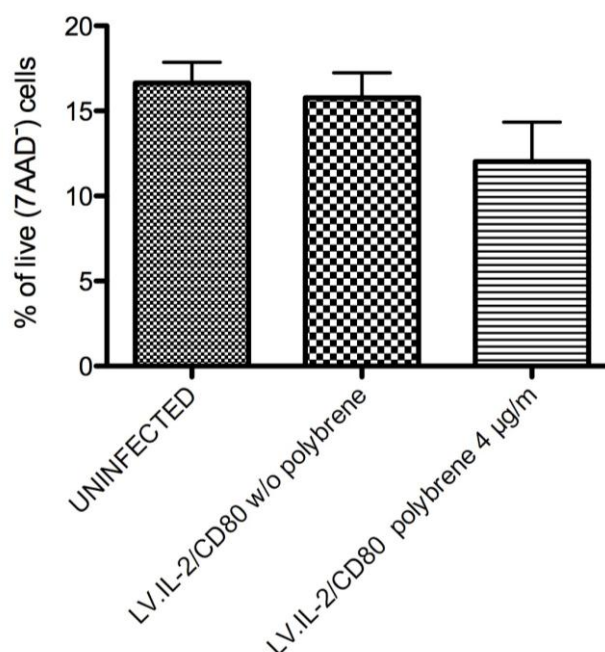


Figure 3-39 Effect of lentiviral transduction primary MM cells viability. CD138⁺ primary MM cells were infected as described in 2.2.2.3.2 at MOI of 20, under standard culture conditions. Cells were then washed twice and cultured for 72 hours prior to analysis of cell viability through 7-AAD staining by flow cytometry. Results represents the means and standard deviation obtained from three replicate wells. Abbreviations: w/o: without.

3.2.3 Discussion

Gene therapy represents a promising strategy for the generation of tumour vaccines able to induce immune responses against cancer. However, this approach has been challenged by the relative inefficiency of gene transfer strategies in haematopoietic cells using retro and adenoviral vectors (Gaken *et al.* 2000), which require the application of multiple rounds of infection as well as the use of selective markers and cell sorting to obtain highly transduced cell populations.

This chapter described the use of third-generation HIV-derived lentiviral vectors in conjunction with strategies to improve the transduction efficiency of target cells, with the aim of obtaining a population of MM cells expressing high levels of CD80 and/or IL-2.

The first version of this vector, concentrated by ultracentrifugation and tested on K562 cells, was able to successfully induce high levels of transgene expression. However, when infecting the MM cell line U266, low levels of transduction and high levels of cytotoxicity were observed. This prompted the evaluation of different strategies for the concentration of the lentiviral vectors and the use of infection enhancers with the aim of increasing the percentage of genetically-modified cells without compromising cell viability. The results showed that the cationic polymer polybrene, already tested in (Lu *et al.* 2007) and (Wen *et al.* 2001a), has a minimal cytotoxic effect on U266 cells when compared to DEAE-dextran (Chan *et al.* 2005a) whilst allowing the transduction of up to 25.5% cells.

In order to compare the stimulatory ability of this vaccination strategy with others based on higher transgene expression levels and to avoid the possibility that the vaccine efficacy could be limited by the proportion of genetically-modified cells, alternative strategies to improve viral presentation to target cells were evaluated. The use of PMP-conjugated lentiviral vectors allowed the concentration of high titers of virus concentration. Additionally, in agreement with previous studies (Chan *et al.* 2005b), the use of PMP increased target cell transduction by promoting additional vector-target interactions. In fact, the fold increase in vector concentration (160 fold, based on the titers calculated when using equivalent volumes of un-concentrated and PMP-

concentrated vectors to infect K562 cells) cannot be completely explained by the fold reduction of the volume of lentivirus resuspension (56 fold), suggesting that this increase is substantially derived from improved viral presentation to target cells. This consideration could not be extended to U266 cells, as a 56 fold reduction in the volume of the lentiviral suspension translated to only a 50 fold increase in virus titer. However, when using equivalent MOI the PMP-concentrated lentiviral vector was able to transduce a higher percentage of cells compared to the vector concentrated by ultracentrifugation, thus supporting the hypothesis that PMP-conjugated vectors may allow additional vector-target cell interactions. Yet, the use of this strategy was limited by the high levels of cytotoxicity associated with the use of high volumes of PMP.

Therefore, with the aim of increasing cell transduction efficiency and, consequently, reducing the amount of PMP-conjugated vector necessary for the infection infection with PMP-conjugated lentiviral vectors was performed on biotinilated cells in the presence of PEI. Although this combination did not succeed in increasing the proportion of genetically-modified cells, the results obtained using increased MOI of PMP-conjugated lentiviral vectors suggested that the use of high titers of virus might alleviate the problematic infection of U266 cells.

The use of a lentiviral vector concentrated by ultracentrifugation at an MOI of 20 was able to induce high levels of transgene expression in U266 cells (68% for LV.IL-2/CD80 and 90.3% for LV.CD80), which were stable for at least 30 days. However, when comparing these results with those described in previous studies (Hirst *et al.* 1997; Wendtner *et al.* 1997; Tarte *et al.* 1999; Wen *et al.* 2001a; Koya *et al.* 2002; Chan *et al.* 2005a; Cignetti *et al.* 2005; Ren *et al.* 2006) it is possible to note two main discrepancies. Firstly, while LV.CD80 is able to induce similar percentages of CD80⁺ cells in both U266 and K562 cells, LV.IL-2/CD80 seems to transduce a significantly lower percentage of U266 cells compared to K562 cells. This result, together with the 5 fold lower secretion of IL-2 obtained when comparing LV.IL-2/CD80 U266 with LV.IL-2 U266 cells, may suggest a low efficiency of the furin endoprotease in these cells. The resulted un-cleaved protein IL-2/CD80 may then not be detected by the anti-CD80 antibody, while

IL-2 would not be secreted. Nevertheless, this phenomenon seems to be cell-line specific as the MM cell line RPMI8226 does not show any difference in transgene expression when infected with equivalent MOI of LV.IL-2/CD80 and LV.CD80.

Secondly, although approximately 90% of U266 cells were successfully genetically-modified when using LV.CD80, two different populations of genetically modified cells could be distinguished on the basis of CD80 expression levels. This phenomenon was again shown to be cell line specific, as LV.CD80 K562 and LV.CD80 RPMI8226 cells presented homogeneous phenotypes in terms of transgene expression. Additionally, it was demonstrated not to be the product of pseudo-transduction or unstable infection as the percentage and MFI of CD80^{high} and CD80^{dim} cells were stable for 30 days. The existence of a heterogeneous U266 cell line was investigated as a potential cause of this phenomenon, but results based on cell granularity, size and phenotypic markers characterisation were not conclusive.

However, despite its reduced permissivity to virus transduction and its unusual transgene expression profile, the U266 cell line was maintained as the target cell for virus transduction owing to its ability to inhibit NK cells (as demonstrated in chapter 3.1) and to its extracellular marker phenotype, which is essential for the design of the functional assay that are used to characterise the effectiveness of this vaccination strategy.

In order to be able to compare the immunomodulatory potential of CD80 and IL-2 with other strategies, genetically-modified U266 cells were successfully sorted on the basis of CD80 expression. It is interesting to note that, even after sorting of CD80⁺ positive cells, IL-2 concentration secreted by LV.IL-2/CD80 U266 cells was still substantially lower than that produced by LV-IL2 U266 cells. However, these levels of secreted proteins could not be compared with the results obtained from other MM genetically-modified cancer vaccines (Wen *et al.* 2001a; Ren *et al.* 2006), as this was the first study using the fusogene strategy and neither of these works evaluated cytokine production from single-gene transduced cells. Nevertheless, these data are in

accordance with those reviewed in Chan (2005a), where they were similarly explained by the reduced rates of transcription, translation and processing of the fusion protein.

Finally, in order to be able to evaluate the advantage of using the combination of CD80 and IL-2, LV.IL-2 U266 cells were mixed with unmodified U266 cells to generate comparable amounts of IL-2 as LV.IL-2/CD80 U266 cells.

Cell viability and phenotype of genetically-modified U266 cells were evaluated and compared with uninfected cells, in particular considering the expression of its ligands CD28 and PD-L1. The results show that infection with lentiviral vectors does not cause a significant change in cell viability (70% live cells for infected and sorted cells compared to 80% live cells for uninfected cells) or in the expression of these markers. The influence of the transduction of MM cells with CD80 and IL-2 on the expression and levels of extracellular markers involved in immunosuppression and cancer survival (such as CTLA-4, Fas and FasL and HLA-I) should be evaluated in future assays.

Finally, following the same protocol used by Cignetti (Cignetti *et al.* 2005) it was possible to successfully infect CD138⁺ cells isolated from a MM patient. Due to the low number of cells available, infection was performed using LV.IL-2/CD80 only. The infection did not cause any major change in cell viability, supporting the feasibility of this strategy using frozen MM plasma cells. Modified cells were characterised by higher levels of CD80 expression compared with Cignetti's and colleagues' results (approximately 94% CD80 positive cells compared to approximately 74%) (Cignetti *et al.* 2005) while secreting lower quantities of IL-2 compared to those obtained using a MM cell line, possibly owing to the low viability of CD138⁺ cells post-thawing. Future experiments will aim to confirm this result using more patient samples and will concentrate on strategies to increase CD138⁺ cells recovery post-thawing and to improve transgene expression in infected MM cells. Additionally, genetically modified cells will be evaluated for any change in the expression of immunomodulatory molecules or receptors involved in tumour survival and cross-talk with the tumour micro-environment.

3.3 Enhanced immune responses to stimulation with IL-2/CD80 genetically-modified cells

3.3.1 Introduction

Having successfully optimised a protocol to genetically-modify myeloma cells, the next step was to test the ability of these genetically-modified cells to act as antigen-presenting cells and to recover immune functions against MM.

Several immunotherapeutic strategies have been tested in recent years for MM. Most of them focused, thanks to the continuous identification of tumour specific antigens and proteins, on targeting idiotypic-specific immunity (as reviewed in 1.4.2.3.1) and on the employment of monoclonal antibodies (as described in 1.4.1.2). While these strategies have been shown in pre-clinical tests to induce anti-tumour effects, the induction of clinically significant response is rare (Danylesko *et al.* 2012) suggesting that their immunomodulatory ability, in the context of a suppressed immune system and inhibitory environment, is too weak to cause significant tumour destruction.

The use of a whole cancer cell vaccine offers the potential for an effective presentation of a broader spectrum of tumour antigens, compared to peptide or protein-based vaccination. In order to counteract the inhibitory activity of myeloma cells, their antigen-presenting ability can be boosted, by inducing the expression of immunomodulatory molecules through genetic manipulation (as outlined in 1.4.2.4.2). Wendtner and colleagues were the first to show the potential of CD80 gene transfer in activating MM cell lysis by allogeneic T and NK cells *in vitro* (Wendtner *et al.* 1997). On the basis of previous studies describing the *in vivo* rejection of CD80-expressing animal tumours (Geldhof *et al.* 1995; Yeh *et al.* 1995), they speculated that this effect could be partially due to NK cells. Tarte and colleagues subsequently confirmed and expanded these results by describing the activation of allogeneic CD8⁺ T-lymphocytes after co-culture with CD80-expressing MM cells (Tarte *et al.* 1999). Following these promising findings, several groups have addressed the potential of different cytokines and receptors to increase CD80-mediated stimulation. Wen and colleagues showed enhanced allogeneic

lympho-proliferation in response to co-culture of PBMCs with CD80/IL-12 modified U266 cells (Wen *et al.* 2001a). Cignetti and Ren, instead, demonstrated the feasibility of lentiviral and retroviral transduction of primary MM cells and that the genetic transfer of CD80-CD40L or CD80-p53-GM-CSF could turn MM cells into efficient APCs, able to expand and activate autologous cytotoxic CD8⁺ cells (Cignetti *et al.* 2005; Ren *et al.* 2006). Finally, in 2007, it was shown that a T cell repertoire recognising myeloma tumour antigens persisted in the peripheral blood of MM patients, and that it could be activated and amplified by autologous tumour cells transduced with CD80 and 4-1BBL (Lu *et al.* 2007).

Due to the controversial role of CD80 in NK cell activation (Geldhof *et al.* 1995; Galea-Lauri *et al.* 1999; Wilson *et al.* 1999; Luque *et al.* 2000; Terrazzano *et al.* 2002), none of these studies evaluated NK cell expansion and function after stimulation of PBMCs with genetically modified myeloma cells. However, Ingram and colleagues (Ingram *et al.* 2009) demonstrated NK cell expansion and activation following co-culture with IL-2/CD80 expressing AML cells.

Given the importance of NK cells in MM control (as outlined in chapter 3.1) and their role in the onset of the adaptive immune response (reviewed in chapter 1.3), this chapter evaluates the immunostimulatory ability of MM cells that are genetically-modified to express CD80 and IL-2 (as described in chapter 3.2), to stimulate the expansion and activation of NK cells. Concurrently, T cell expansion and activation was also characterised.

This immunomodulatory strategy presents several advantages compared with those previously experimented (reviewed in chapter 1.3). Firstly, the choice of two immunomodulatory molecules whose importance in the context of anti-tumour immune response has been widely established: the co-stimulatory molecule CD80, which is poorly expressed on MM cell surface and has been shown to be down-regulated on DCs from MM patients, and the pro-inflammatory cytokine IL-2, which is known for its important role in the activation of NK and CTL responses. The combination of these molecules has already been proven to be effective in stimulating T and NK cell functions

in the context of AML (Chan *et al.* 2005a; Ingram *et al.* 2009) and its safety and efficacy is currently being evaluated in a Phase I clinical trial.

A second potential improvement relies on the choice of a self-inactivating lentiviral vector coupled with the fusogene strategy for the expression of CD80 and IL-2, whose advantages in terms of safety and efficient cell transduction have been discussed in Chapter 3.2. Finally, this study examines NK cell activation following stimulation with genetically-modified myeloma cells, in the context of the biological mechanisms involved in MM-induced inhibition of NK cells, as shown and described in chapter 3.1.

The results reviewed in this chapter show that the allogeneic stimulation of healthy donor PBMCs with IL-2/CD80 expressing MM cells is able to expand NK and T cell numbers, and to induce a significant increase in the fraction of NK cells expressing the activating receptors NKp44, NKp30 and CD69, when compared to unmodified MM cells. This was also substantiated by data obtained when using PBMCs isolated from MM patients.

Finally and more importantly for potential therapeutic application of this strategy, NK cells from healthy donors stimulated with IL-2/CD80 modified MM cells showed increased cytolytic activity against unmodified MM cells.

As previous studies have shown the potential of IMiDs to activate the immune system and, in particular, NK cells (Schey *et al.* 2004; Harrison and Cook 2005), this immunomodulatory strategy was combined with IMiDs such as pomalidomide and lenalidomide in order to evaluate potential beneficial synergistic effects. In particular, given the indication that IMiDs co-stimulate T cells via the CD80-CD28 pathway (LeBlanc *et al.* 2004) experiments have been performed with the aim of comparing the ability of the IMiDs pomalidomide and lenalidomide to activate and restore NK cell functions against MM, either alone or in combination with the IL-2/CD80 whole cell vaccines. However, the protocol used in this study failed to show any synergistic effect between IMiDs and IL-2/CD80 cell vaccine.

3.3.2 Results

3.3.2.1 Set-up of optimal conditions for co-culture of PBMCs and genetically-modified MM cells

Genetically-modified MM cells (generated as described in chapter 3.2) were used to stimulate PBMCs isolated from healthy donors and MM patients. However, in order to comply with the biosafety standards, modified tumour cells were required to be gamma-irradiated before administration. Therefore, the optimal amount of radiation needed to stop cell proliferation was evaluated. As shown in figure 3-40, 50 grays (Gy) constitutes a sufficient dose of radiation to ensure the arrest of cell proliferation.

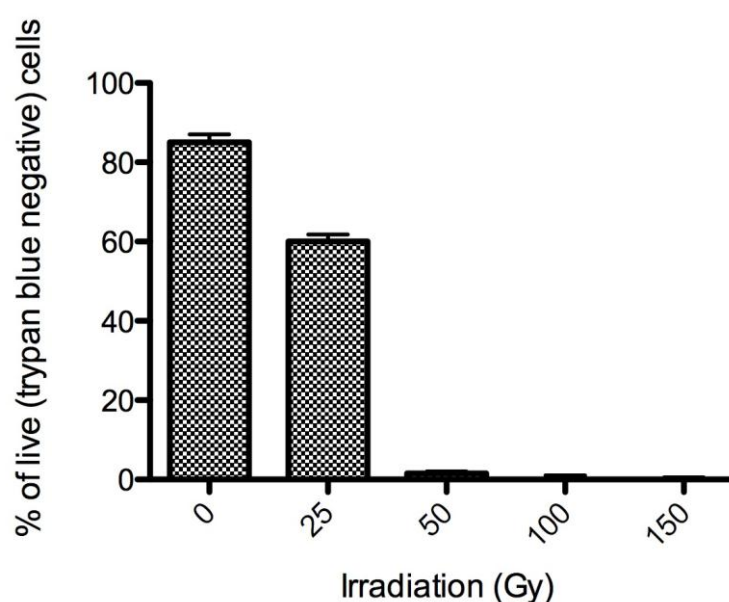


Figure 3-40 LV.IL-2/CD80 U266 cells viability after irradiation.

LV.IL-2/CD80 U266 cells were γ -irradiated using different doses of radiation (Gy). Seven days after irradiation, cells were harvested and a trypan-blue based cell viability count was performed. Bars represent the mean and standard deviation from replicate wells.

Following irradiation, cells were also monitored for CD80 and IL-2 expression for 7 days, which represents the maximal duration of co-culture tested in previous works (Wendtner *et al.* 1997; Wen *et al.* 2001a; Chan *et al.* 2005a; Cignetti *et al.* 2005; Ren *et al.* 2006; Lu *et al.* 2007; Ingram *et al.* 2009). The results illustrated in figure 3-41 demonstrate that CD80 continues to be expressed until day 5. After this time, it is no longer possible to detect the presence of live cells.

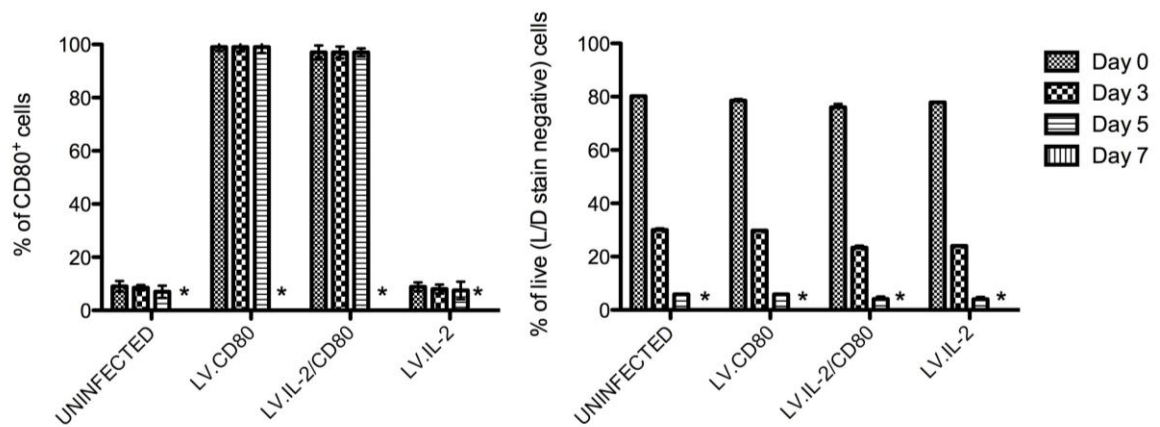


Figure 3-41 CD80 expression of LV.-modified and uninfected cells after irradiation. Cells were γ -irradiated at 50 Gy. Cells were then harvested at the indicated time points and tested for CD80 expression (left) and viability (right) through the use of a live/dead (L/D) stain by flow cytometry. CD80 expression was calculated as percentage of live/dead stain negative U266 cells. * indicates results below the detection limit of the instrument. Bars represent the mean and standard deviation from replicate wells.

Figure 3-42 shows that IL-2 continues to accumulate in the medium until the end of the timeframe tested.

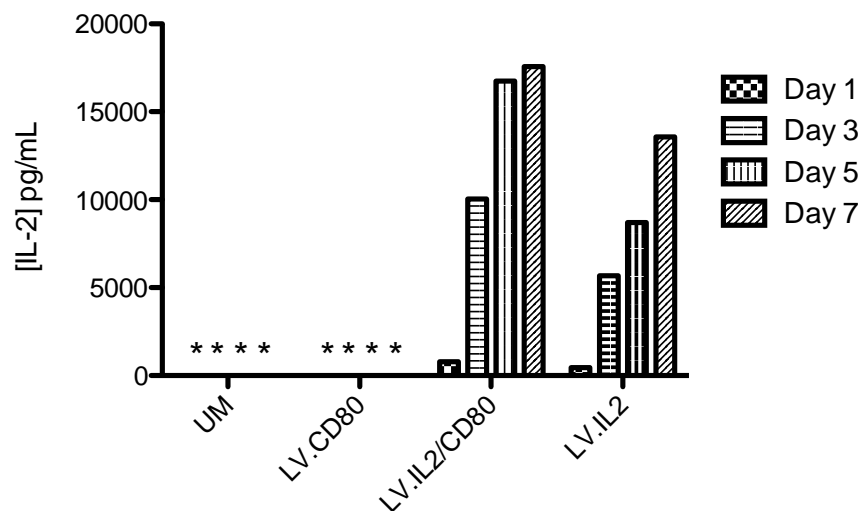


Figure 3-42 IL-2 production after irradiation by LV.IL-2/CD80 U266 cells. LV.IL-2/CD80 U266 cells were seeded at 5×10^5 cells/mL and γ -irradiated at 50 Gy. Supernatants were harvested at the indicated time points and stored at -20°C . At the end of the co-culture period, supernatants were thawed and analysed for IL-2 concentration. * indicates results below the detection limit of the instrument. Bars represent the mean and standard deviation from replicate wells.

To identify the most suitable co-culture period and evaluate NK cell activation in response to stimulation with genetically-modified cells, a time-course experiment was performed. Healthy donor PBMCs were co-cultured with unmodified or LV.IL-2/CD80 modified U266 cells, and NK cell numbers and the expression of activating receptors (Ingram *et al.* 2009) were evaluated at day 0, day 1, day 3, day 5 and day 7 by flow cytometry. The indicated time points were chosen on the basis of co-culture periods adopted in previous studies (Wen *et al.* 2001a; Chan *et al.* 2005a; Cignetti *et al.* 2005; Ren *et al.* 2006; Lu *et al.* 2007; Ingram *et al.* 2009).

As shown in Figure 3-43, NK cell numbers and expression of activating receptors appear to reach a peak around day 5 and day 7. This support the hypothesis that day 7 is an appropriate time point to evaluate NK cell responses to stimulation by LV.IL-2/CD80 U266 cells. Of all the receptors investigated, NKp44 and CD69 showed the greatest increase. Although CD69 expression plateaued at day 3, its levels remained higher than those observed after co-culture with un-modified cells on day 5 and 7 as well. When evaluating DNAM-1, NKG2D and NKp46, no differences were detected between the stimulatory ability of LV.IL-2/CD80 U266 cells and that of unmodified cells.

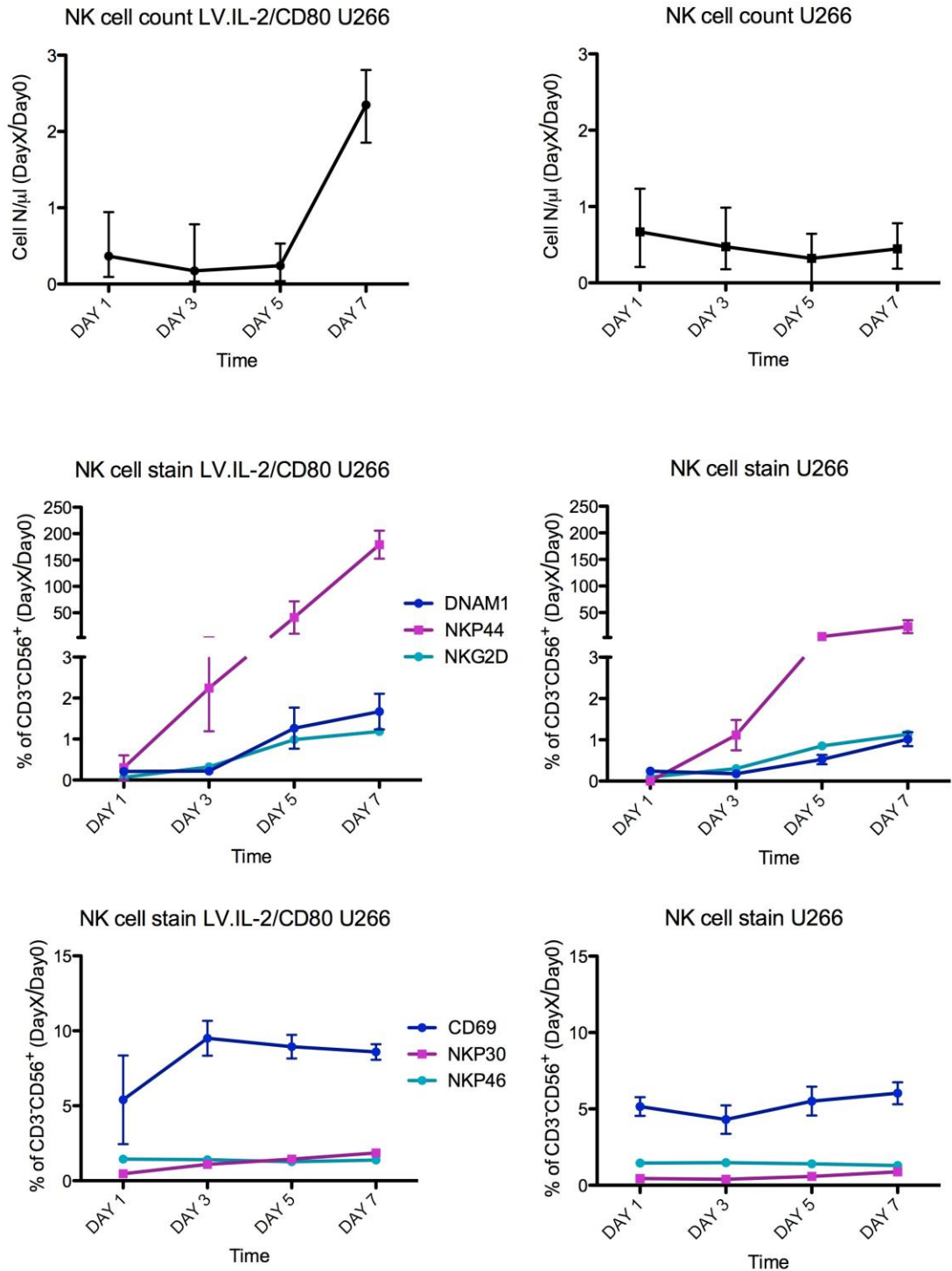


Figure 3-43 Effect of *in vitro* co-culture of healthy donor PBMCs with unmodified U266 (right column) or IL-2/CD80 cells (left column) on NK cell numbers and phenotype. NK cell numbers and phenotype were determined as described in 2.5.3.1 and 2.5.3.2 on day 0 and at different time points following co-culture with modified or unmodified U266 cells at a ratio of 2:1. The median and range from two healthy donors are shown as fold increase over Day 0.

3.3.2.2 Allogeneic stimulation of healthy donor (HD) PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the *in vitro* expansion and activation of NK cells

In order to prove that genetically-modified MM cells are better stimulators compared with their unmodified counterparts, their ability to stimulate NK cell proliferation was investigated. Concurrently, it was also evaluated whether simultaneous expression of both transgenes (CD80 and IL-2) would result in increased stimulation compared to single transgene expression.

As shown in figure 3-44, the presence of unmodified U266 cells in the co-culture alone induced the expansion of NK cells, which increased from 11.7 cells/ μ L, when PBMCs were cultured in medium alone, to 44.8 cells/ μ L. Interestingly, although the expression of these immunomodulatory molecules did further boost NK cell numbers to 58.4 cells/ μ L, in response to LV.CD80 U266 cells, and to 58.34 cells/ μ L, in response to LV.IL-2 U266 cells, this increase reached statistical significance only when comparing it with LV.IL-2/CD80 modified cells, which stimulated NK cell proliferation to 91.3 cells/ μ L ($P \leq 0.01$).

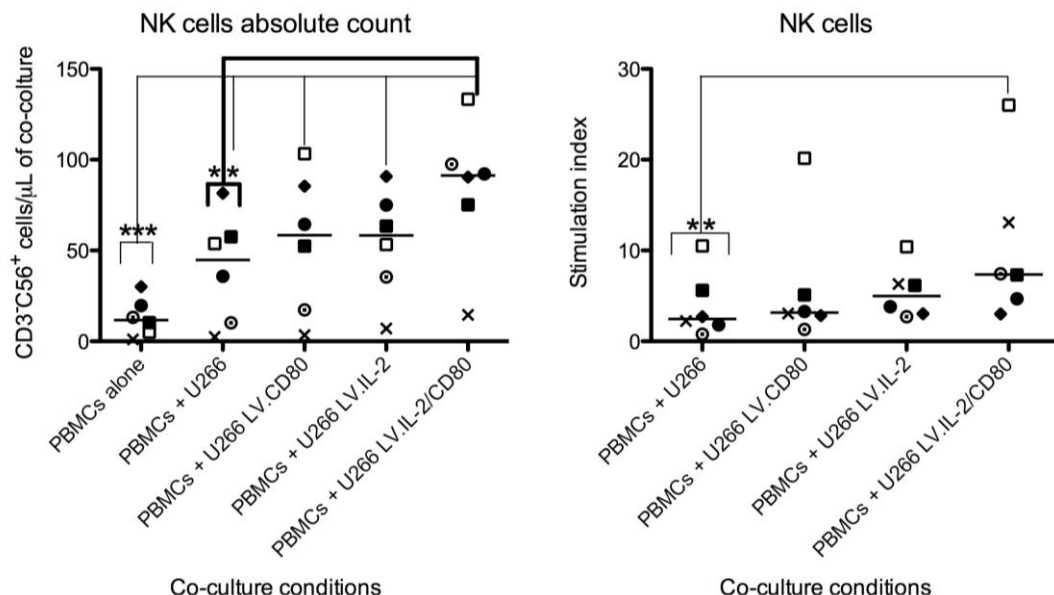


Figure 3-44 Effect of *in vitro* co-culture of HD PBMCs with un-modified or modified U266 cells on NK cell numbers.

Healthy donor PBMCs were co-cultured either with complete medium alone or in the presence of un-modified or LV.CD80, LV.IL-2 and LV.IL-2/CD80 U266 cells. The absolute number (left) and the stimulation index (right, calculated as increase over unstimulated PBMCs) of NK cells were determined after 7 day co-culture as described in 2.5.3.1. The plots illustrate the distribution and the grand median for each of the conditions performed on seven HD. ** $P \leq 0.01$; ***, $P \leq 0.001$.

The antibody panel developed in chapter 3.1 to describe MM-induced downregulation of NCRs was subsequently used to evaluate changes in the expression of these receptors on NK cells after co-culture with unmodified or genetically-modified U266 cells. However, following previous findings (Figure 3-43), NKp46 was not considered as a suitable marker. As for NKG2D, NKp30 and DNAM-1, the decision to include them in the panel was made based on their importance in MM cell recognition and killing by NK cells (Chapter 3.1).

Figure 3-45 shows that, of all the receptors screened, only NKp44, CD69 and NKp30 were upregulated in the presence of modified U266 cells. In particular, a statistically significant increase in the expression of NKp44 was detected when comparing the stimulatory ability of LV.IL-2/CD80 U266 cells, characterised by a median of 57.05% NKp44⁺ cells, with that of unmodified cells, which showed a median of 17.26% NKp44⁺ cells ($P \leq 0.01$). The percentage of CD69 expressing NK cells increased from 21.65% in unstimulated PBMCs, to 78.8% and 83% CD69⁺ cells after stimulation with LV.IL-2 U266 and LV.IL-2/CD80 U266 cells respectively ($P \leq 0.05$). Similar increases were reported when testing NKp30 expression, from 25.2% in the presence of unmodified U266 to 70.6% and 70.4% NKp30⁺ cells when PBMCs were co-cultured with LV.IL-2 U266 and LV.IL-2/CD80 U266 cells respectively ($P \leq 0.05$). In agreement with preliminary data (Figure 3-43), no significant change in DNAM-1 or NKG2D expression was observed.

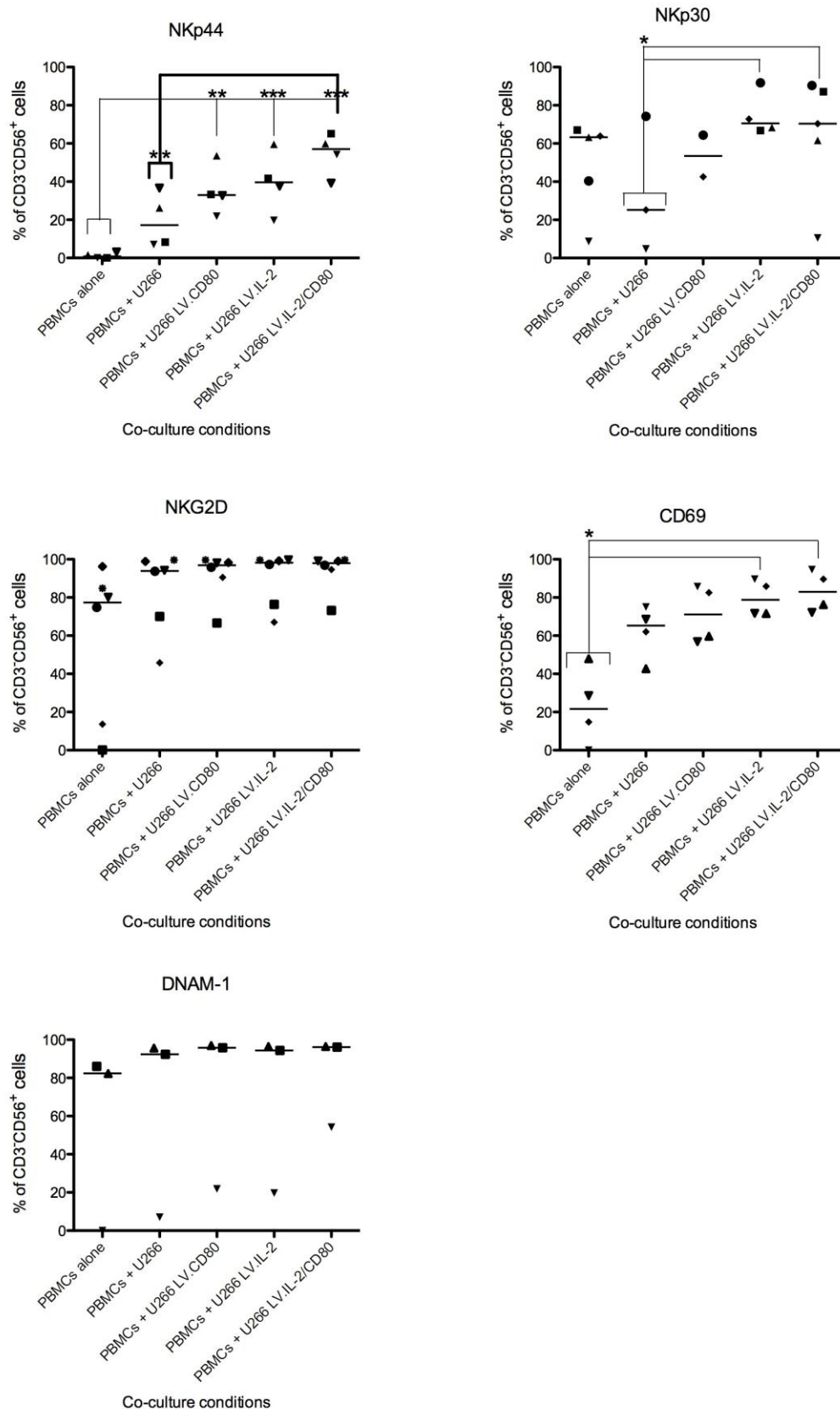


Figure 3-45 Effect of *in vitro* co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on NCR expression.

Healthy donor (HD) PBMCs were co-cultured as described in Figure 3-44. The percentage of expression for each NCR on CD3⁺CD56⁺ NK cells was examined after 7 days by flow cytometry. Plots illustrate the distribution and the grand median for each of the conditions performed on seven HD * $P \leq 0.05$, ** $P \leq 0.01$; ***, $P \leq 0.001$

A parallel increase in NK cell cytolytic activity following co-culture with genetically-modified cells was next investigated. Unmodified U266 cells were chosen as target cells in this assay as one of the aims of this vaccination strategy is to increase NK cell cytotoxicity against unmodified cells. The results show that co-cultures with LV.IL-2 and LV.IL-2/CD80 U266 cells were able to significantly increase NK cytolytic activity towards unmodified U266 cells from a median of 31.8% 7-AAD⁺ U266 cells in the presence of unmodified stimulator U266 cells to a median of 49.7% 7-AAD⁺ U266 cells in the presence of LV.IL-2 U266 ($P \leq 0.05$) and 54.26% 7-AAD⁺ U266 cells in the presence of LV.IL-2/CD80 ($P \leq 0.01$). Although it was not possible to show a statistically significant difference between LV.IL-2 and LV.IL-2/CD80 modified cells, the trends reported in Figure 3-46 suggest that the combination of these factors provide a better stimulation compared to IL-2 alone.

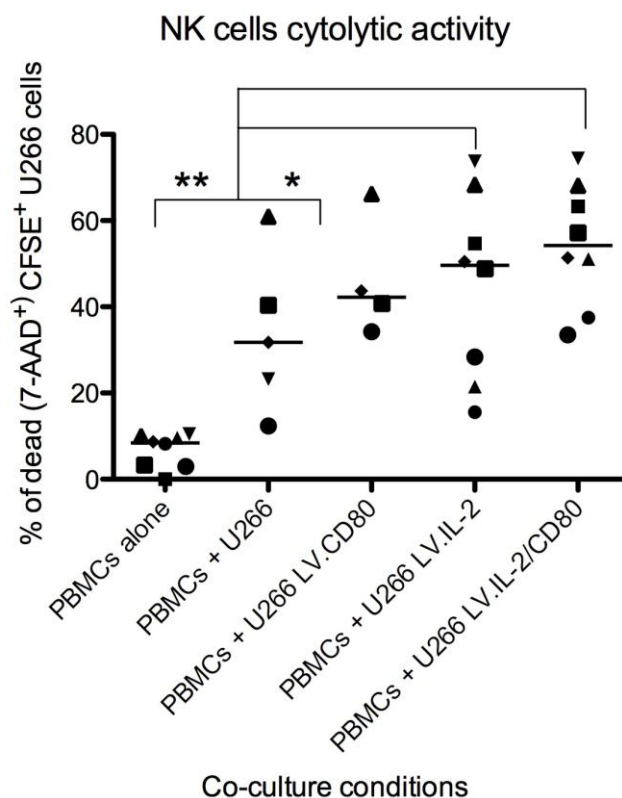


Figure 3-46 Effect of *in vitro* co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on NK cell cytolytic activity towards unmodified U266. Healthy donor PBMCs were co-cultured as described in Figure 3-44. After 7 days NK cells were purified and tested for cytolytic activity against U266 target cells at an effector to target ratio of 5 to 1. Results represent the distribution and the grand median for each of the conditions performed. * $P \leq 0.05$; **, $P \leq 0.01$.

3.3.2.3 Allogeneic stimulation of HD PBMCs with IL-2/CD80 genetically-modified U266 MM cells induces the *in vitro* expansion and activation of T cells

Although not the primary aim of this investigation, the stimulatory ability of genetically-modified cells on T cells was evaluated following allogeneic co-culture. This interest arose from the important role of CD80 in T cell stimulation (Allison, 1994), from the evidence of T cell expansion and activation reported in similar studies (Tarte *et al.* 1999; Wen *et al.* 2001a; Chan *et al.* 2005a; Cignetti *et al.* 2005; Ren *et al.* 2006; Lu *et al.* 2007) and from the need to assess the potential role and influence of this activation on the NK cell phenotype and activity following co-culture with genetically-modified MM cells. After co-culture with LV.CD80 and LV.IL-2/CD80 U266 cells, the number of CD3⁺ T cells increased from a median of 61 cells/ μ L to 153 and 119 respectively ($P \leq 0.05$). This corresponds to stimulation indices of 1.9 and 2.3 when compared to untreated PBMCs. The presence of IL-2 alone, instead, did not seem to contribute to T cell expansion (Figure 3-47).

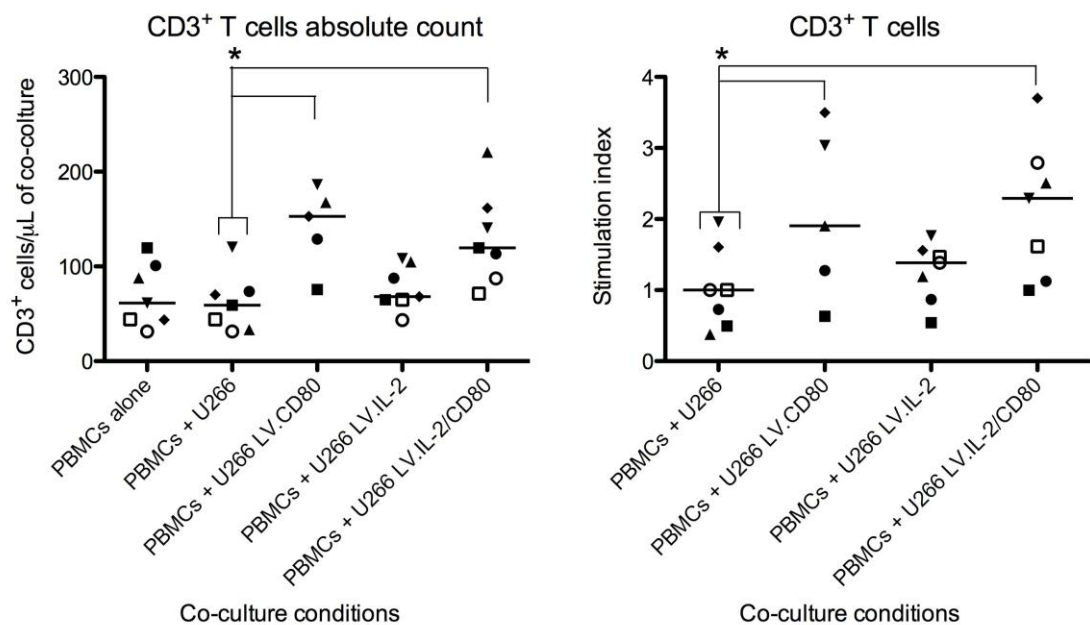


Figure 3-47 Effect of *in vitro* co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on T cell numbers.

Healthy donor PBMCs were co-cultured either as described in Figure 3-44. The absolute number (left) and the stimulation index (right, calculated as increase over unstimulated PBMCs) of T cells, defined as CD138⁺CD3⁺CD56⁺ cells, were determined after 7 days. Plots illustrate the distribution and the grand median for each of the conditions performed on seven HD. * $P \leq 0.05$.

CD8⁺ T cells were next evaluated for the expression of the activation markers HLA-DR and CD69 (Figure 3-48), chosen as late and early activation markers for T cells (Speiser *et al.* 2001; Chan *et al.* 2005a). No statistically significant difference between the different treatments was measured when considering CD69 expression, whereas the stimulation with LV.CD80 and LV.IL-2/CD80 U266 cells induced a significant up-regulation of HLA-DR. The median of HLA-DR⁺CD8⁺ T cells, in fact, rose from 30.7% in the presence of unmodified cells, to 52.5% and 47.9% when PBMCs were stimulated with LV.CD80 and LV.IL-2/CD80 U266 cells respectively ($P \leq 0.05$).

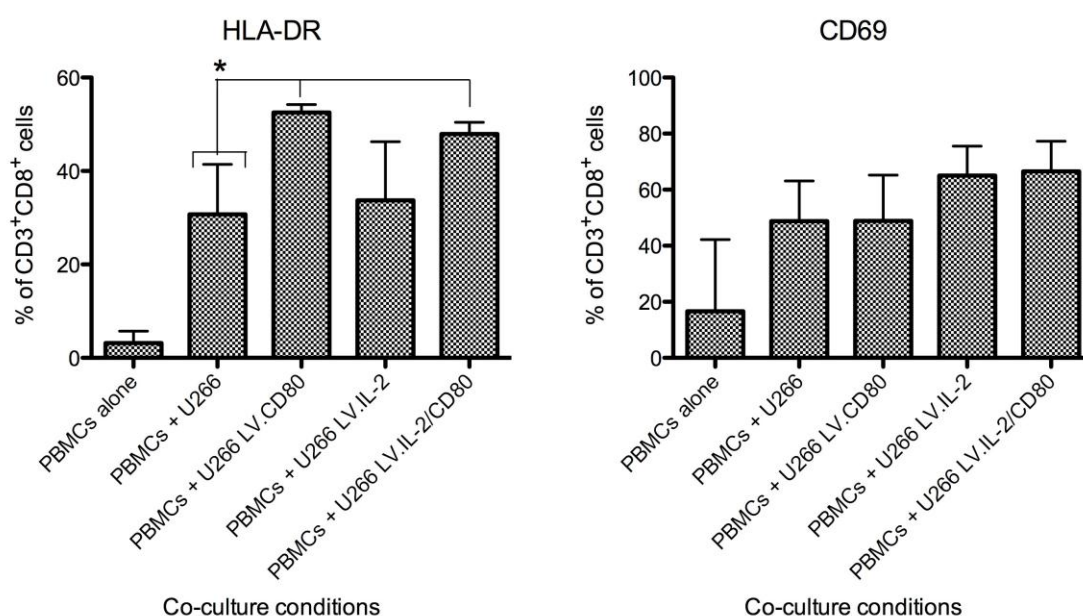


Figure 3-48 Effect of *in vitro* co-culture of healthy donor (HD) PBMCs with un-modified or modified U266 cells on CD8⁺ T cells HLA-DR and CD69 expression. Healthy donor PBMCs were co-cultured as described in Figure 3-44. The percentage of CD69 and HLA-DR positive CD8⁺ T cells was examined after 7 days by flow cytometry. Bars illustrate the median and range for each of the conditions performed on four healthy donors. * $P \leq 0.05$.

3.3.2.4 IMiDs (Pomalidomide, Lenalidomide) fails to show synergy with IL-2/CD80 genetically-modified U266 MM cells for the induction of NK cell cytolytic activity in Healthy Donors

IMiDs such as pomalidomide and lenalidomide have been subsequently tested for their ability to activate and restore NK cell functions against MM, either alone or in combination with the IL-2/CD80 whole cell vaccines.

However, experiments aiming to identify the best dose of IMiDs, in terms of concentration and frequency of treatment, failed to confirm the results previously obtained in the literature (Figure 3-49 and 3-50).

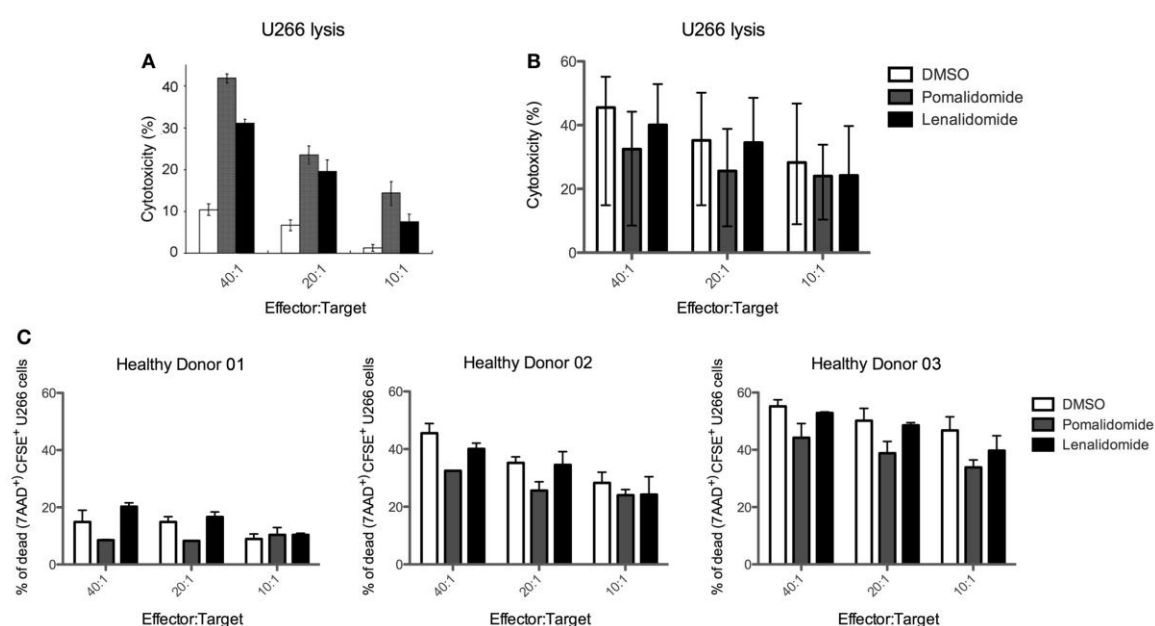


Figure 3-49 Effect of *in vitro* co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against U266 cells.

NK cell activity after stimulation with IMiDs (B) was compared with results obtained by Hayashi (Hayashi *et al.* 2005) (A). Healthy donor PBMCs were co-cultured with either medium alone or in the presence of IMiDs (1 μ M). After 5 days PBMCs were tested for cytolytic activity against U266 (B) target cells at different effector to target ratios. Graph B represent the median and range obtained from three healthy donors. Results from singular donors are shown in graphs C.

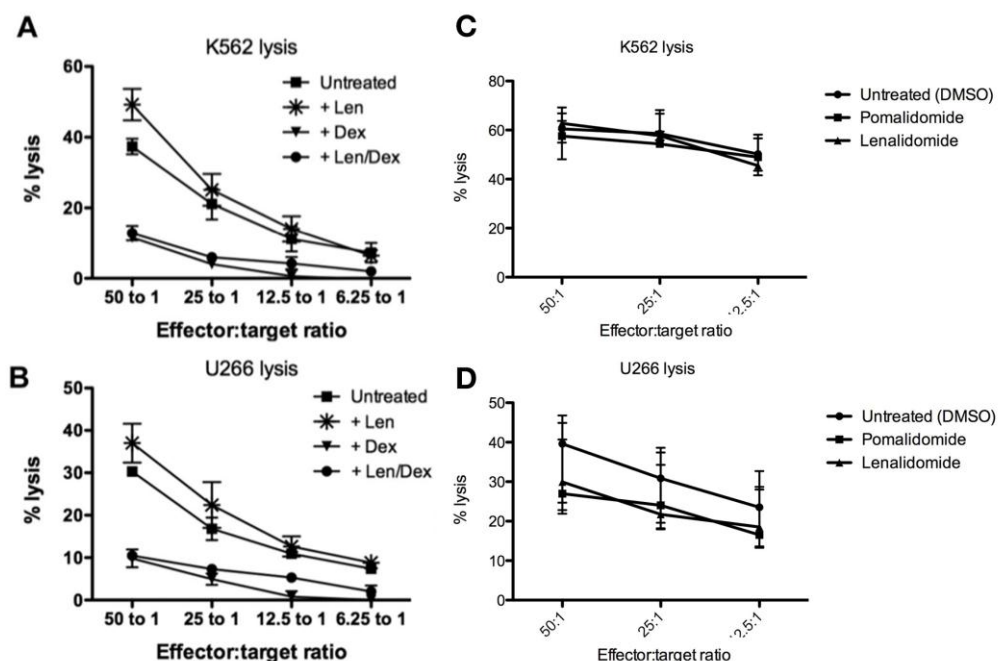


Figure 3-50 Effect of *in vitro* co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against K562 and U266 cells. NK cell activity after stimulation with IMiDs (C-D) was compared with results obtained by Hsu (Hsu *et al.* 2011) (A-B). Healthy donor PBMCs were co-cultured with either medium alone or in the presence of IMiDs (10 μ M). After 3 days PBMCs were tested for cytolytic activity against K562 (C) or U266 (D) target cells at different effector to target ratios. Results represent the median and range obtained from three healthy donors.

In subsequent assay, it was possible to observe a trend towards an increase in NK cell cytolytic activity when treating healthy donor PBMCs with pomalidomide (3 μ M) for 3 days (Figure 3-51), but this increase did not reach statistical significance.

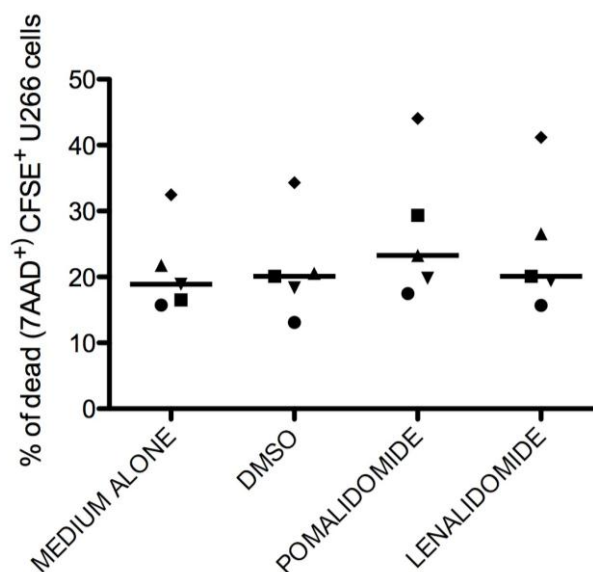


Figure 3-51 Effect of *in vitro* co-culture of healthy donor PBMCs with IMiDs on NK cell cytolytic activity against U266 MM cells. Healthy donor PBMCs were co-cultured with either medium alone or in the presence of IMiDs (3 μ M). After 3 days PBMCs were tested for cytolytic activity against U266 target cells at an effector to target ratio of 50:1. Results represent the median obtained from five healthy donors.

In a pilot experiment, this drug concentration was subsequently administered in conjunction with genetically-modified U266 cells but failed to show synergy with CD80/IL-2 expressing myeloma cells for the induction of NK cell cytolytic activity (Figure 3-52).

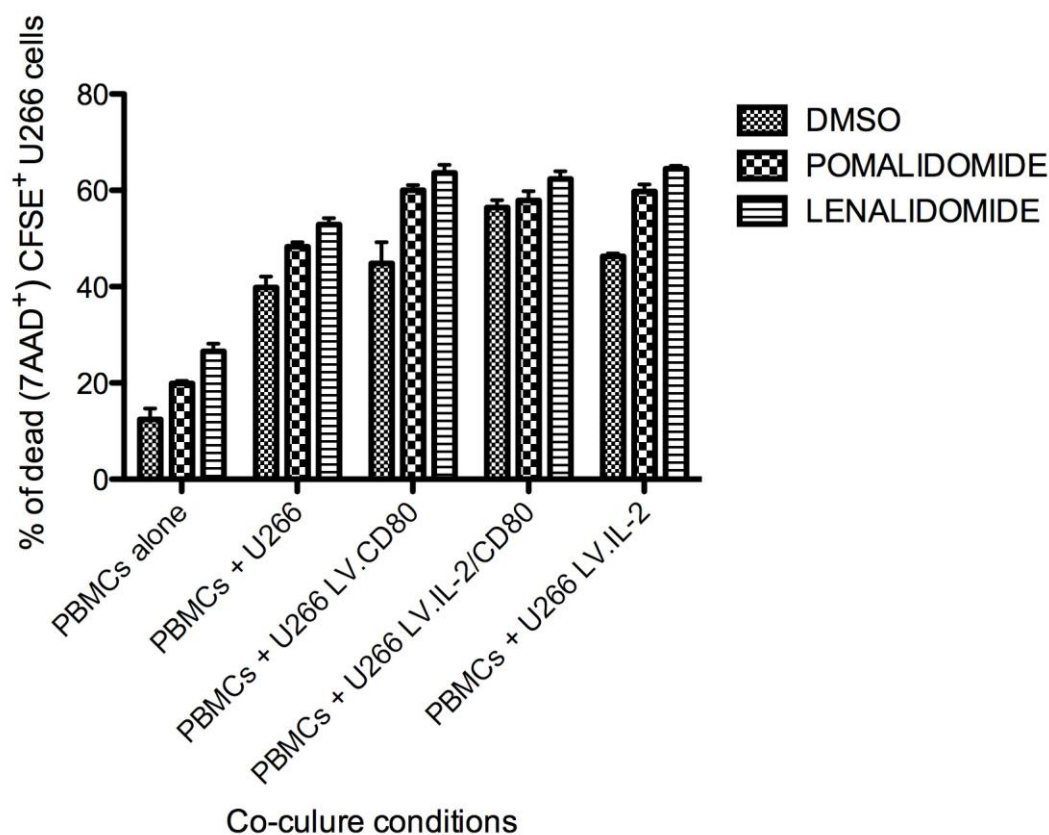


Figure 3-52 Effect of *in vitro* co-culture of healthy donor PBMCs with un-modified or modified U266 cells on NK cell cytolytic activity against unmodified U266. Healthy donor PBMCs were co-cultured with either medium alone or in the presence of un-modified or LV.CD80, LV. IL-2 and LV.IL-2/CD80 U266 cells. On day 2, IMiDs or DMSO were added at a final concentration of 5 μ M. After 7 days NK cells were purified and tested for cytolytic activity against U266 target cells at an effector to target ration of 5 to 1. Results represent the mean with standard deviation of triplicate wells from one healthy donor.

3.3.2.5 Allogeneic stimulation of MM patient PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the *in vitro* expansion of NK cells

The stimulatory ability of genetically-modified U266 cells on PBMCs from MM patients was next investigated. *De novo* patient samples were used to minimise the introduction of confounding variables such as the treatment with immunomodulatory drugs, which may interfere with this immunostimulatory strategy. A total of 9 patients were considered but, due to the limited numbers of available PBMCs, only 4 sets of cultures were stimulated with unmodified and genetically-modified cells. Patient characteristics are summarised in Table 2-6.

Due to patient-to-patient variability in the number of NK cells retrieved after a 7 days co-culture in complete medium alone, the results are reported as fold increase over unstimulated PBMCs, defined as stimulation index. The data show that co-culture of MM patient PBMCs with LV.IL-2/CD80 U266 cells induced a statistically significant increase in the stimulation index of NK cell, which rose from 484.7 in the presence of unmodified U266 cells to 1147 when PBMCs were co-cultured with LV.IL-2/CD80 U266 cells ($P \leq 0.05$) (Figure 3.53).

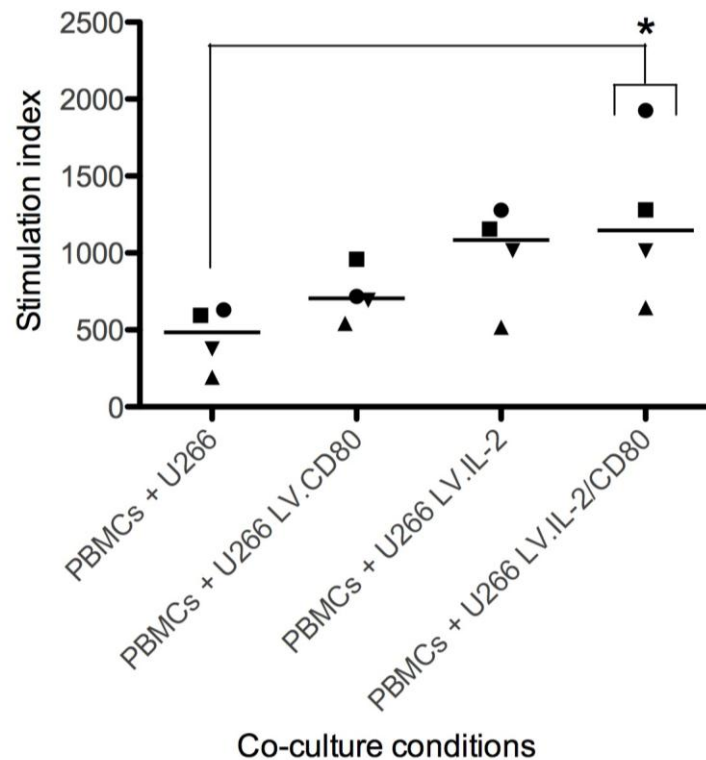


Figure 3-53 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on NK cell numbers.

PBMCs from MM patients were co-cultured in the presence of un-modified or LV.CD80, LV. IL-2 and LV.IL-2/CD80 U266 cells for 7 days. The stimulation index of NK cell numbers was determined after 7 days. Plots illustrate the distribution and the grand median for each of the conditions performed on four MM patient samples. * $P \leq 0.05$.

The close proximity of the stimulation indices obtained following co-culture with LV.IL-2 and LV.IL-2/CD80 U266 cells suggests the important role of IL-2 in NK cell proliferation. The importance of CD80 remains unclear, although it is possible to note an increase in NK cell numbers when MM patient PBMCs were stimulated with LV.CD80 U266 cells, which lead to a stimulation index of 705 compared the 484.7 obtained in the presence of unmodified U266 cells. This effect, although diminished, is still visible when comparing the fold increase in the presence of LV.IL-2 (1085.9) and LV.IL-2/CD80 U266 cells (1147).

NK cells were also tested for the upregulation of NCR in response to stimulation with unmodified and genetically modified U266 cells. As the number of NK cells retrieved after a 7 day co-culture with medium alone did not provide enough events to perform a flow cytometry phenotypic analysis, this condition was not considered in the context of this assay.

The limited number of patients tested did not provide enough data to evaluate the significance of these differences (Figure 3-54). However, it is possible to observe a trend towards an increase in the percentage of NKp30 expressing NK cells, which increased from a median of 39.9% in the presence of unmodified cells to 88.6% when PBMCs were co-cultured with LV.IL-2/CD80 U266 cells. Similar increases were reported when testing NKp44 expression, from 63.5% in the presence of unmodified U266 to 87.5% NKp44⁺ cells when LV.IL-2/CD80 U266 cells were used as stimulator cells.

NK cells were also tested for the mean fluorescent intensity (MFI) of these receptors, to determine whether genetically-modified cells might have an effect on their levels of expression, but no significant changes were observed (Figure 3-55).

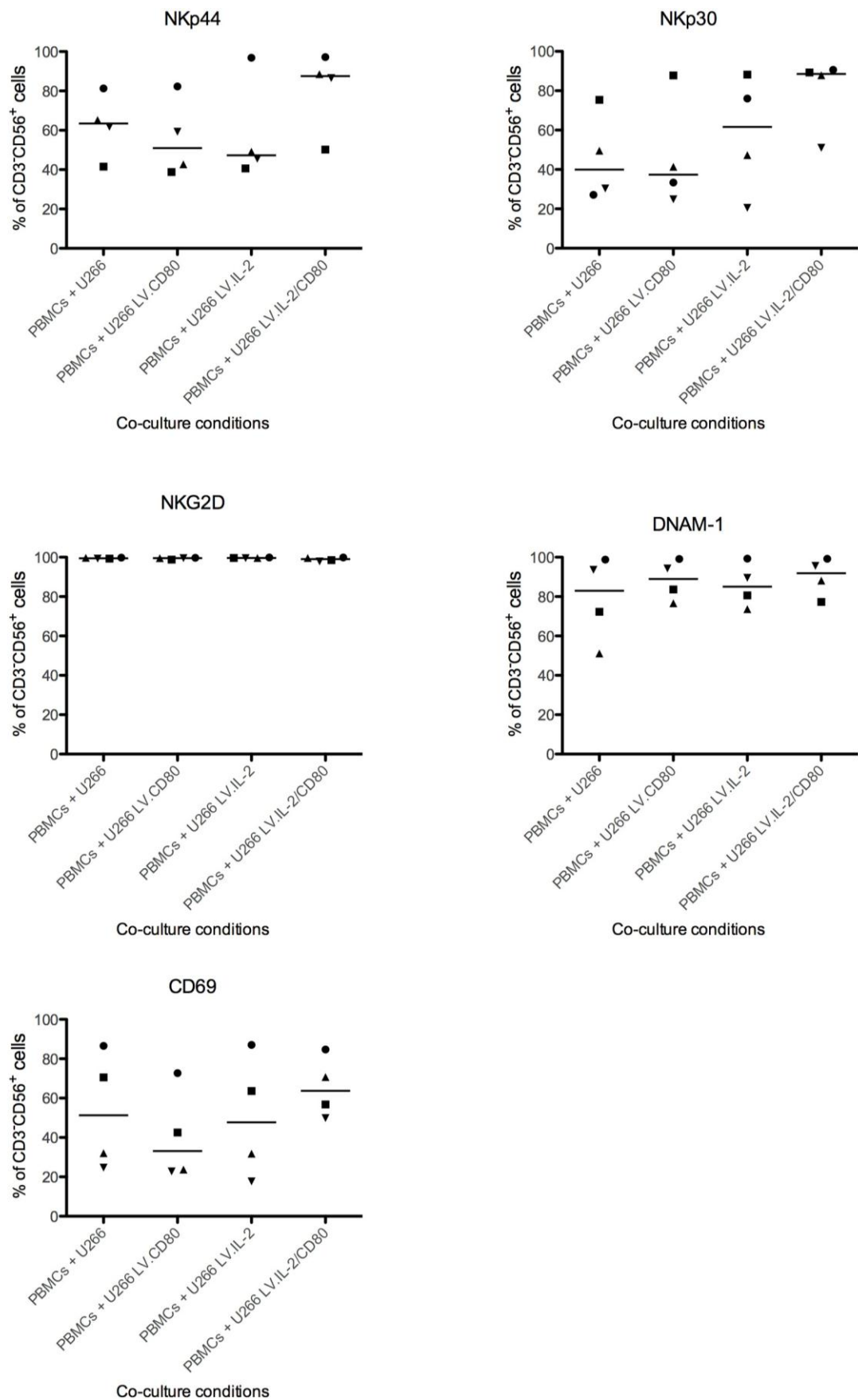


Figure 3-54 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on NCR expression.

PBMCs from MM patients were cultured as described in Figure 3-53. The expression of NCRs was examined after 7 days by flow cytometry. Plots illustrate the distribution and the grand median for each of the conditions performed on four MM patient samples

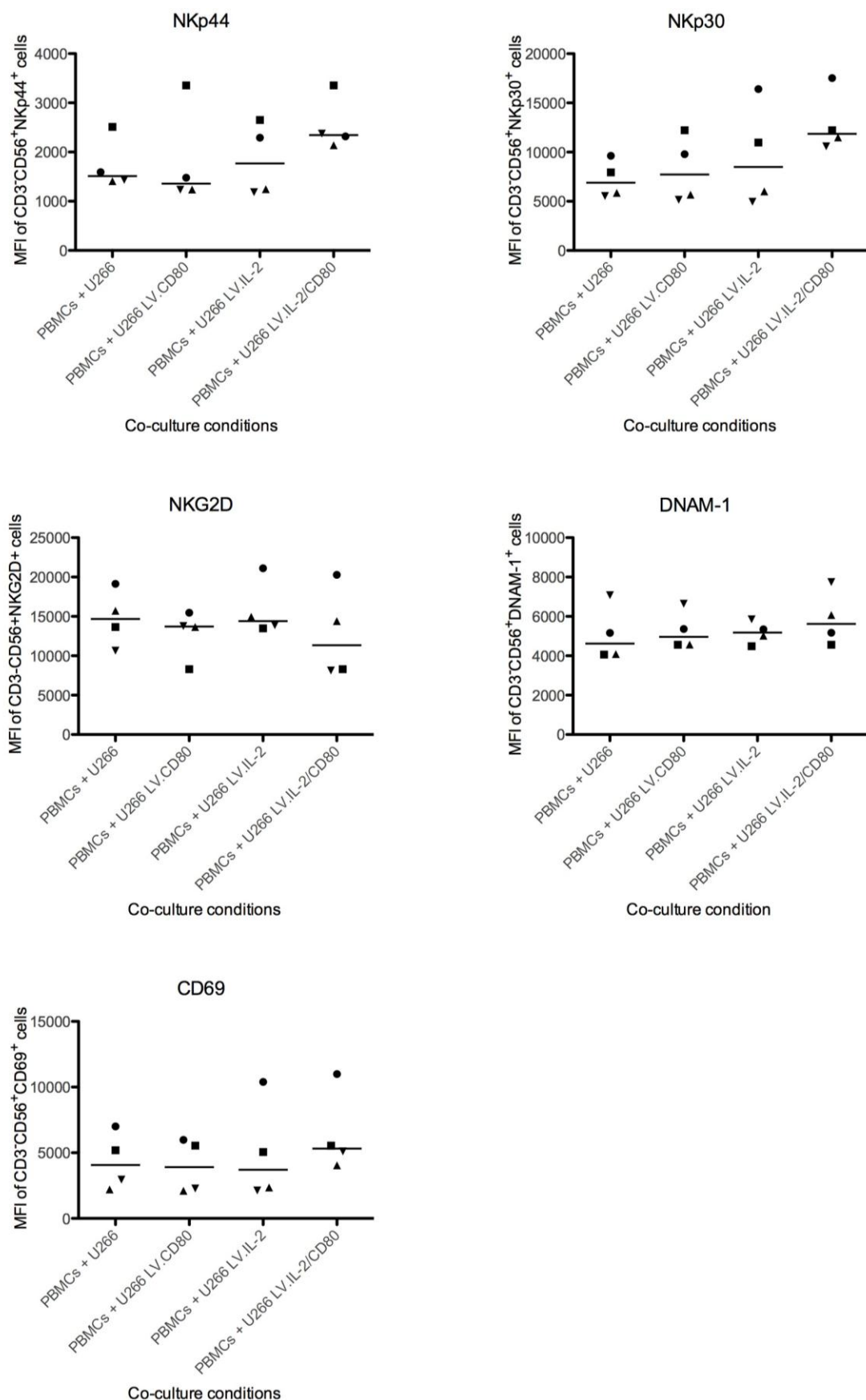


Figure 3-55 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on NCR expression levels.

PBMCs from MM patients were cultured as described in Figure 3-53. The mean fluorescence intensity (MFI) for each NCR was examined after 7 days by flow cytometry. Plots illustrate the distribution and the grand median for each of the conditions performed on four MM patient samples.

Finally, the cytolytic activity of stimulated NK cells was tested against unmodified U266 cells (Figure 3-56).

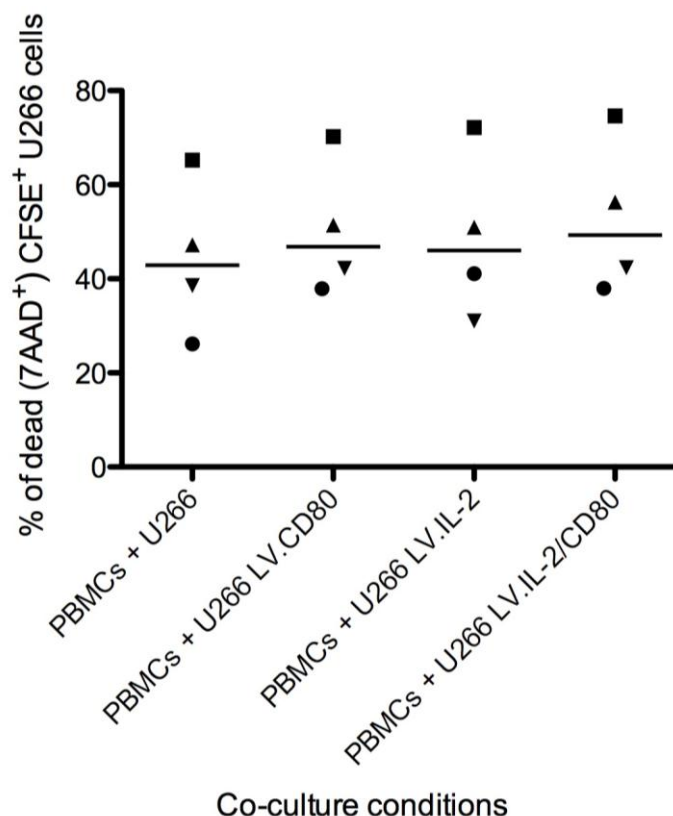


Figure 3-56 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on NK cell cytolytic activity towards unmodified U266 cells. PBMCs from MM patients were cultured as described in Figure 3-53. After 7 days NK cells were purified and tested for their cytolytic activity against U266 target cells at an effector to target ration of 5 to 1. Results represent the distribution and the grand median for each of the conditions performed on four MM patient samples.

The results show a high cytolytic activity of NK cells after co-culture with unmodified U266 (42.9% of 7-AAD⁺U266 cells), which was not significantly increased by the co-culture of MM patient PBMCs with genetically-modified U266.

An alternative *in vitro* assay was therefore employed as an additional method to investigate NK cell activation. This was based on the evaluation of NK cell degranulation through the measurement of CD107a expression on the cell surface and IFN- γ secretion levels. However, as shown in Figure 3-57, the results obtained were consistent with those observed using the 7-AAD based-killing assay, as there was no indication of a statistically significant difference either in the percentage or absolute levels of expression of CD107a or IFN- γ following co-culture with unmodified or genetically-modified U266 cells.

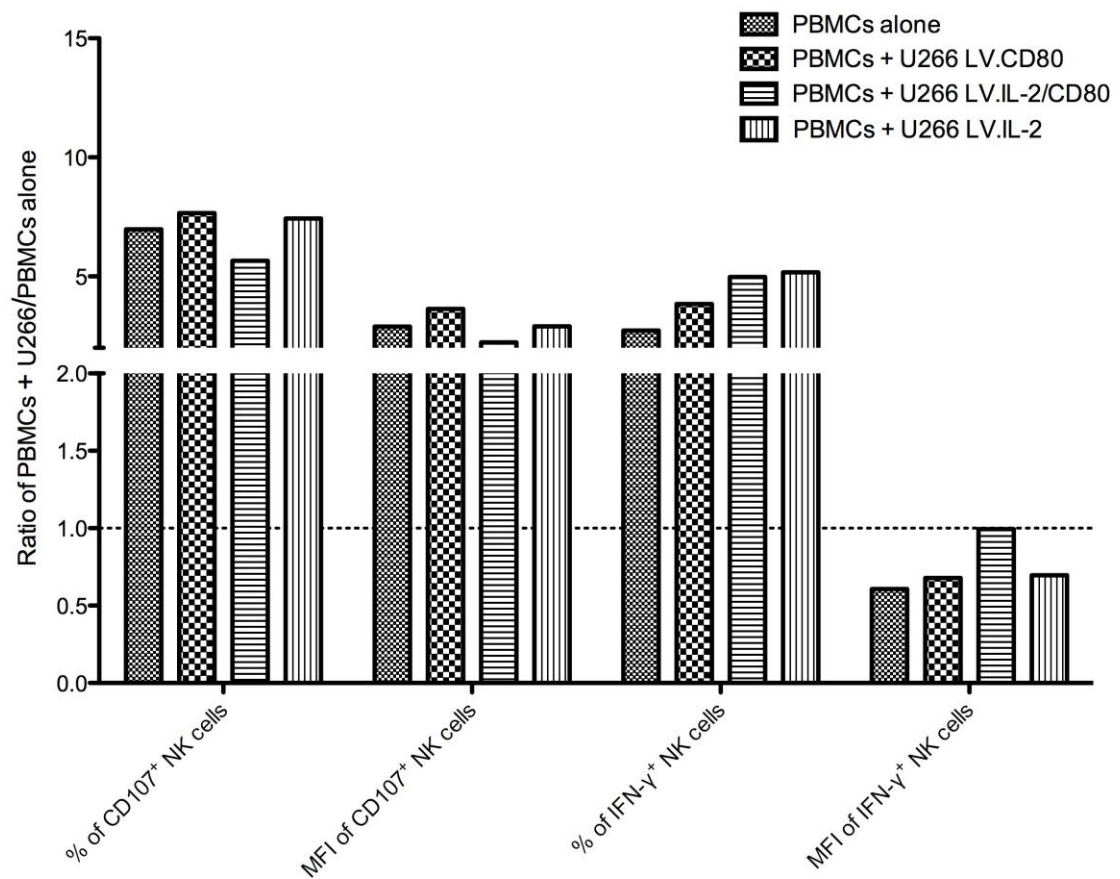


Figure 3-57 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on NK cell degranulation and IFN- γ secretion levels.

PBMCs isolated from MM patients were co-cultured either with medium alone or in the presence of un-modified or LV.CD80, LV. IL-2 and LV.IL-2/CD80 U266 cells. After 7 days PBMCs were harvested and incubated with X-VIVO 15 complete medium alone or U266 cells at an effector to target ratio of 1 to 1. CD107a and IFN- γ expressions were examined by flow cytometry as described in 2.5.4.2. Bars represent fold increase over unchallenged PBMCs performed on one MM patient.

3.3.2.6 Allogeneic stimulation of MM patient PBMCs with IL-2/CD80 genetically-modified U266 MM cells allows the *in vitro* expansion of T cells

The effect of the co-culture of MM patient PBMCs with genetically-modified cells on T cell populations was next investigated. The co-culture of MM patient PBMCs with genetically-modified U266 cells induced the proliferation of CD3⁺ and further analysis indicated this was due mainly to the CD8⁺CD3⁺ cell compartment (Figure 3-58). However, statistical significance was only reached when comparing PBMCs co-cultured in the presence of unmodified or LV.IL-2/CD80 U266 cells ($P \leq 0.05$). No statistically significant difference was observed when comparing CD4⁺ T cell numbers following the different stimulation strategies.

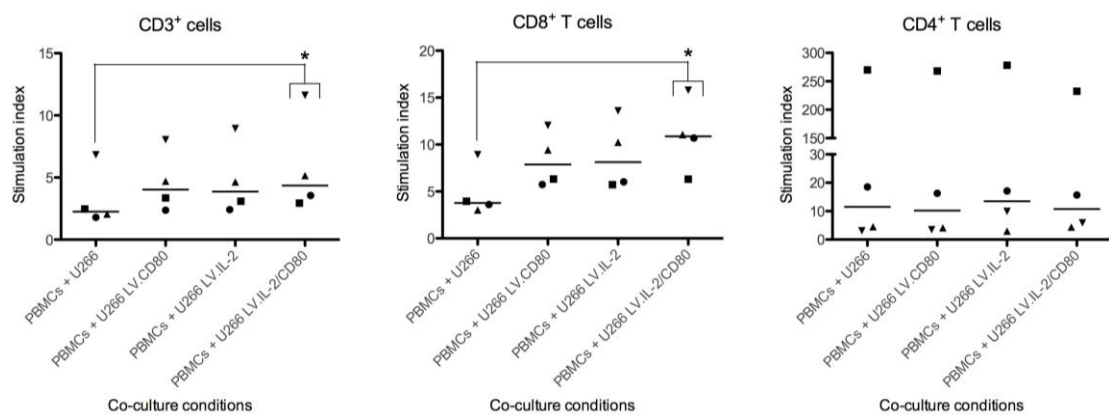


Figure 3-58 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on T cell numbers. PBMCs isolated from MM patients were cultured with medium alone (unstimulated PBMCs) or in the presence of un-modified or LV.CD80, LV. IL-2 and LV.IL-2/CD80 U266 cells. Stimulation indices (calculated as increase over unstimulated PBMCs) for CD3⁺ T cells (CD138⁺CD3⁺CD56⁺), CD8⁺ T cells (CD8⁺CD4⁺ T cells) and CD4⁺ T cells (CD8⁺CD4⁺ T cells) were determined by flow cytometry on day 7 following *in vitro* culture as indicated. Plots illustrate the distribution and the grand median for each of the conditions performed on four MM patients. * $P \leq 0.05$.

When investigating the expression of activating receptors on CD8⁺T cells, CD154 was added to the antibody panel as it is a marker of late T cell activation (Mackey *et al.* 1998). The results reported in Figure 3-59 show a trend towards the up-regulation of HLA-DR and CD69 expression when MM patient PBMCs are co-cultured in the presence of LV.CD80 and LV.IL-2/CD80 modified U266 cells. Although it is possible to see a trend toward an increase in CD154 expression levels (MFI) following stimulation with LV.IL-2/CD80 U266 cells, this did not reach significant statistical difference.

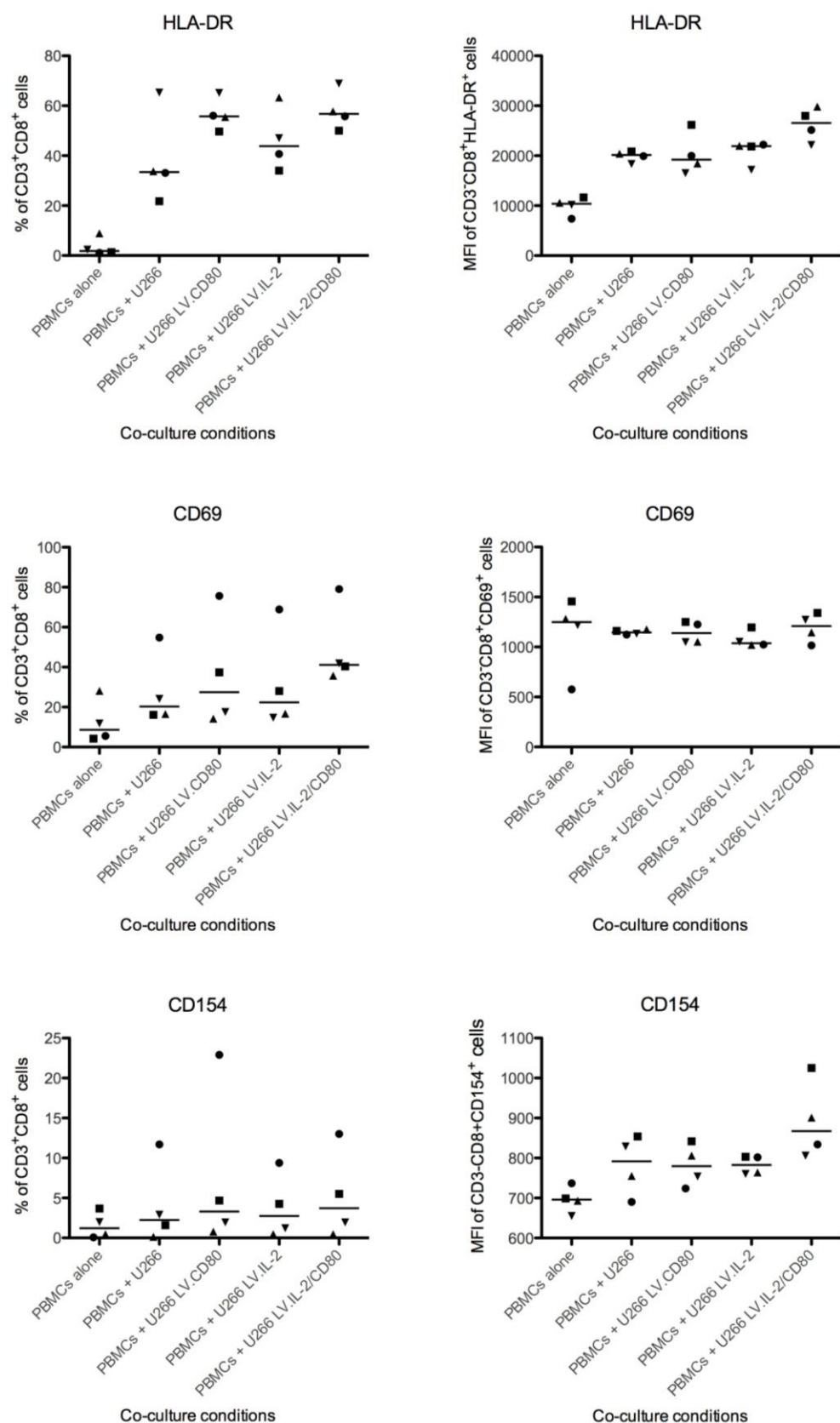


Figure 3-59 Effect of *in vitro* co-culture of MM patient PBMCs with un-modified or modified U266 cells on CD8⁺ T cell receptor expression.

PBMCs from MM patients were cultured as described in Figure 3-58. The percentage of positive cells and expression levels (MFI) for each receptor were examined at day 7 by flow cytometry. Plots illustrate the distribution and the grand median for each of the conditions performed on four MM patients.

3.3.3 Discussion

Vaccination of MM patients with newly identified multiple MM antigens represents an interesting immunotherapeutic strategy, but the number of “universal” MM antigens presently known is limited (Pellat-Deceunynck, 2003). Vaccination with whole tumour cells might then represents an attractive alternative. The use of malignant cells as antigen presenting cells has, in fact, the potential to allow the effective presentation of both shared and unique tumour antigens.

After investigating the molecular mechanisms underlying MM-induced NK cells inhibition and highlighting a link between downregulation of Natural Cytotoxicity Receptors (NCR) and suppression of NK cell cytolytic activity (Chapter 3.1), a MM cell line was successfully genetically-modified with a lentiviral vector to express IL-2 and CD80, with the aim of stimulating NK cell function against MM (Chapter 3.2).

The results illustrated in this chapter demonstrate that NK cell activity can be enhanced following allogeneic stimulation with genetically-modified MM cells.

Specifically, a 7 day allogeneic co-culture of healthy donor PBMCs with LV.IL-2/CD80 U266 cells resulted in a statistically significant expansion of NK cells, compared to unmodified U266 cells. The data shown in this chapter also suggest a role for CD80 in the increase in NK cell numbers, as noted when comparing LV.IL-2 and LV.IL-2/CD80 U266 cells as stimulators. Nevertheless, the difference did not reach statistical significance, possibly reflecting the predominant role of IL-2 in NK cell activation. A larger cohort of samples is needed to provide additional data to support or reject this hypothesis.

The expanded NK cells also demonstrated an activated phenotype, as they expressed increased levels of the activating receptors NKp44, NKp30 and CD69. Some of the trends observed in the kinetics of NCR up-regulation suggest the possibility to retrieve higher levels of proliferation and activation after day 7. This would be in agreement with the results obtained by Chan (Chan *et al.* 2005a), Ren (Ren *et al.* 2006), (Tarte *et al.* 1999), Wen (Wen *et al.* 2001a), Cignetti (Cignetti *et al.* 2005) and Lu (Lu *et al.* 2007), who demonstrated the activation of T cells after multiple rounds of stimulation.

However, considering that NK cells, by their definition, can efficiently be activated without prior stimulation and on the basis of the results obtained in a similar AML setting after one round of stimulation (Ingram *et al.* 2009), it was decided to adopt 7 days as the incubation period for subsequent assays. It was also encouraging to observe that the fold increase in NCR expression is similar to that seen by Ingram (Ingram *et al.* 2009) when using IL-2/CD80-modified AML cells. Additionally, in this MM setting, the up-regulation in the expression of NKp44 and NKp30 was observed not only when comparing un-stimulated and LV.IL2/CD80 stimulated PBMCs, as described by Ingram (Ingram *et al.* 2009), but also when comparing modified and unmodified U266 cells. This observation supports the role of CD80 and IL-2 stimulation in NK cell activation.

The downregulation of NKp30 following co-culture with unmodified U266 cells confirms the data presented in Chapter 3.1. The ability of genetically-modified U266 cells to recover its expression to levels comparable with healthy donor PBMCs is therefore important, particularly in the light of the role of NKp30 in MM-induced inhibition of NK cells (Chapter 3.1). However, no statistically significant difference was measured between the stimulatory ability of LV.IL-2 and LV.IL-2/CD80 U266 cells suggesting, once again, a predominant role for IL-2 in NK cell activation. A similar increase in the expression of CD69 was induced by co-culture of PBMCs with LV.IL-2 and LV.IL-2/CD80 U266 cells, which is in agreement with the role of IL-2 in the up-regulation of this receptor (Borrego *et al.* 1999). Finally, it was not possible to demonstrate any change in DNAM-1 and NKG2D expression following co-culture with genetically-modified or unmodified cells. This could be a result of the kinetics of up-regulation of these receptors, suggesting that their levels may plateau after a 3 day co-culture or an impact of allogeneic reactions on their expression.

LV.IL-2/CD80 genetically modified U266 cells showed the ability to activate MM patients NK cell expansion. However, when evaluating the up-regulation of NCRs on the expanded NK cells, the data did not reach statistical significance. Further experiments using a larger cohort of patients are needed to confirm these observations.

Importantly in the light of a possible clinical application of this strategy, the

stimulation with LV.IL-2/CD80 and LV.IL-2 modified U266 cells appears to confer the greatest augmentation of cytolytic activity against unmodified U266 cells. This result confirms the data published by Ingram (Ingram *et al.* 2009) in an AML setting but, in addition, show an increase in NK cell cytolytic activity following co-culture with LV.CD80 U266 cells. The increase of cytolytic activity due to the presence of CD80 is also supported by the trend towards an increase in the levels of U266 cell death induced by PBMCs following stimulation with LV.IL-2/CD80 compared to LV.IL-2 U266 cells. However, this difference did not reach statistical significance and further studies are needed to establish the precise role and possible interplay of CD80 and IL-2 in NK cell activation.

No significant change was observed in NK cell activity following co-culture of MM patient PBMCs with unmodified or genetically-modified U266 cells. However, in contrast to the results reported by Ingram (Ingram *et al.* 2009), high levels of background in NK cell activation were observed after 7 day co-culture with unmodified MM cells. This result was not expected considering the functional inhibition which characterises NK cells from MM patients (described in Chapter 3.1), but might be a consequence of a strong response to the allogeneic co-culture, or of the need for a shorter or longer co-culture period. Further studies should be performed to clarify the causes underlying this phenomenon.

As CD80 is mainly known as a co-stimulatory molecule for T cells, T cell expansion and phenotype following allogeneic co-culture were also evaluated. The aim was to determine whether the observed NK cell activation could be the consequential to T cell stimulation by the vaccine. The results showed that, as expected, stimulation of healthy donor PBMCs with CD80-expressing MM cells induced the expansion of CD3⁺ T cells. However, published data have reported different test methods and variable stimulator to responder ratios, making it difficult to compare stimulation indexes. Nevertheless, the results from co-cultures of MM patient PBMCs with LV.IL-2/CD80 U266 cells showed a trend towards an increase in CD3⁺ cell numbers, which was mostly due to the proliferation in the CD8 compartment. This is in line with findings obtained by Tarte

(Tarte *et al.* 1999) and Chan (Chan *et al.* 2005a) and suggests an important role for CD80 in T cell expansion and activation, as previously shown using CD80 expressing AML (Chan *et al.* 2005a) and MM cells (Tarte *et al.* 1999; Wen *et al.* 2001a; Cignetti *et al.* 2005; Ren *et al.* 2006; Lu *et al.* 2007). CD8⁺ T cells from co-cultures of both healthy donor and MM patient PBMCs, also presented higher levels of expression of the activation marker HLA-DR when LV.CD80 and LV.IL-2/CD80 U266 cells were used as stimulators. However, overall, the combination of CD80 and IL-2 was not proven to be significantly better than the stimulation by CD80 alone. Further studies considering multiple rounds of stimulation of PBMCs with genetically-modified cells and the functional characterisation of the stimulated T cells by IFN- γ and Granzyme B ELISPOT technique should be performed.

Our results so far failed to demonstrate any effect of IMiDs, either alone or in combination with IL-2/CD80 modified MM cells, on NK cell activity. This was mainly due to the high background levels of NK cell cytolytic activity in the presence of DMSO, which was used to resuspend the drugs. Future studies should aim to optimise lenalidomide and pomalidomide treatment protocol on healthy donor PBMCs, possibly starting with sourcing the drug from alternative suppliers. The optimised protocol should then be tested on PBMCs from MM patients.

Chapter 4 Concluding remarks

In vitro and *in vivo* cytolytic activity of NK cells against MM cells has been widely demonstrated (Godfrey and Benson 2012) (and reviewed in Chapter 1.2). However, NK cell-based immunotherapy has resulted in limited clinical benefit, possibly reflecting the mechanisms that tumour cells have developed to avoid NK cell-mediated recognition and killing (Pietra *et al.* 2012). Progress in the understanding of these mechanisms has the potential to lead to new opportunities to recover or augment NK cell function as therapeutic interventions for the treatment of MM.

This thesis provides new data on the molecular mechanisms responsible for the MM-mediated inhibition of NK cells and how to recover and activate NK cell functions against tumour cells.

The results presented in Chapter 3.1 supports the importance of contact-dependent interactions and the role of the activating NK receptors NKG2D, DNAM-1 and NKp30 in the control and recognition of MM cells. The requirement for different receptors, shown here and in the literature in various tumour settings may be suggestive of immune selection events that, following direct cell-to-cell interactions, have shaped the tumour and NK cell surface phenotype. Future experiments using molecules that disrupt receptor-ligand interactions, such as Fc-proteins, should further investigate the role of NK cell receptors in MM-induced inhibition of NK cells. In this context, these studies should also aim to correlate the inhibitory activity of different MM cell lines with their phenotypical characteristics.

Due to the importance of NKp30, NKG2D and NKp46 on NK-cell induced maturation of DC and the killing of immature DCs by NK cells, the downregulation of

these receptors is expected to have an impact not only on NK cells, but also on the shaping and modulation of the adaptive response. Therefore, future studies should aim to evaluate the effect of inhibited NK cells on DC and T cell functions. These data have the potential to help define new therapeutic targets and therapeutic guidelines for MM patients, according to their immune and tumour phenotypes.

In view of this immunosuppressive effect, an immunomodulating strategy to prevent and/or rescue myeloma-induced suppression of immuno-mediated responses was subsequently evaluated (Chapter 3.3).

The studies described in Chapter 3.2 illustrated that a third-generation lentiviral vector is able to successfully and stably modify MM cells to express the immunomodulatory molecules CD80 and IL-2. Preliminary results obtained using frozen CD138⁺ plasma cells from one MM patient support the feasibility of this procedure. Future studies should validate these results and optimise the cell thawing, culture and infection procedures in order to increase viability and expand the primary MM cell. This will be crucial with the aim of obtaining sufficient numbers as required for *in vivo* vaccination strategies.

The results illustrated in Chapter 3.3, finally showed that IL-2/CD80 genetically-modified U266 cells generated in Chapter 3.2 enhance NK and T cell expansion and activation when used to stimulate both healthy donor and patient PBMCs. The contribution of CD80 and IL-2 alone on NK and T cell activation should be further investigated to determine their stimulatory potential and with the view to explore the potential benefit of other cytokine combinations. Furthermore, new experiments using purified NK cells as targets of the stimulation should establish whether NK cell activation is the result of a direct or indirect stimulation by the genetically-modified cells. In this regard, the suggested activation of T cells following stimulation with LV.IL-2/CD80 MM cells should be further examined through functional assays such as IFN- γ and Granzyme ELISPOT on pure or sorted T cell populations.

Due to the limited number of MM patient screened, it is difficult to make any conclusion regarding the therapeutic potential of this whole cancer cell vaccine.

Therefore, a larger cohort of MM patients (n=30) should be employed. Future studies should also consider evaluating the effect of this immunomodulatory strategy using a more heterogeneous group of MM patients both in terms of disease status and treatment.

The results from these experiments will help evaluating the potential benefit of this strategy for patients in active disease, stable disease and relapse and outline possible synergistic effect with other therapeutic options such as IMiDs.

Bibliography

- Alici, E., T. Sutlu, B. Bjorkstrand, M. Gilljam, B. Stellan, H. Nahi, H. C. Quezada, G. Gahrton, H. G. Ljunggren and M. S. Dilber (2008). "Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components." Blood **111**(6): 3155-3162.
- Alter, G., J. M. Malenfant and M. Altfeld (2004). "CD107a as a functional marker for the identification of natural killer cell activity." J Immunol Methods **294**(1-2): 15-22.
- Alyea, E., E. Weller, R. Schlossman, C. Canning, I. Webb, D. Doss, P. Mauch, K. Marcus, D. Fisher, A. Freeman, B. Parikh, J. Gribben, R. Soiffer, J. Ritz and K. Anderson (2001). "T-cell-depleted allogeneic bone marrow transplantation followed by donor lymphocyte infusion in patients with multiple myeloma: induction of graft-versus-myeloma effect." Blood **98**(4): 934-939.
- Andersson, S., C. Fauriat, J. A. Malmberg, H. G. Ljunggren and K. J. Malmberg (2009). "KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression." Blood **114**(1): 95-104.
- Antonia, S. J., J. Seigne, J. Diaz, C. Muro-Cacho, M. Extermann, M. J. Farmelo, M. Friberg, M. Alsarraj, J. J. Mahany, J. Pow-Sang, A. Cantor and W. Janssen (2002). "Phase I trial of a B7-1 (CD80) gene modified autologous tumor cell vaccine in combination with systemic interleukin-2 in patients with metastatic renal cell carcinoma." J Urol **167**(5): 1995-2000.
- Azuma, M., M. Cayabyab, D. Buck, J. H. Phillips and L. L. Lanier (1992). "CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes." J Exp Med **175**(2): 353-360.
- Bajenoff, M., B. Breart, A. Y. Huang, H. Qi, J. Cazareth, V. M. Braud, R. N. Germain and N. Glaichenhaus (2006). "Natural killer cell behavior in lymph nodes revealed by static and real-time imaging." J Exp Med **203**(3): 619-631.
- Balsamo, M., F. Scordamaglia, G. Pietra, C. Manzini, C. Cantoni, M. Boitano, P. Queirolo, W. Vermi, F. Facchetti, A. Moretta, L. Moretta, M. C. Mingari and M. Vitale (2009). "Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity." Proc Natl Acad Sci U S A **106**(49): 20847-20852.
- Barry, S. C., J. Seppen, N. Ramesh, J. L. Foster, K. Seyama, H. D. Ochs, J. V. Garcia and W. R. Osborne (2000). "Lentiviral and murine retroviral transduction of T cells for expression of human CD40 ligand." Hum Gene Ther **11**(2): 323-332.
- Bashirova, A. A., M. P. Martin, D. W. McVicar and M. Carrington (2006). "The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense." Annu Rev Genomics Hum Genet **7**: 277-300.
- Bataille, R., G. Jigo, N. Robillard, S. Barille-Nion, J. L. Harousseau, P. Moreau, M. Amiot and C. Pellat-Deceunynck (2006). "The phenotype of normal, reactive and malignant plasma cells. Identification of "many and multiple myelomas" and of new targets for myeloma therapy." Haematologica **91**(9): 1234-1240.
- Baume, D. M., M. J. Robertson, H. Levine, T. J. Manley, P. W. Schow and J. Ritz (1992). "Differential responses to interleukin 2 define functionally distinct subsets of human natural killer cells." Eur J Immunol **22**(1): 1-6.
- Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik and T. J. Schall (1997). "A new class of membrane-bound chemokine with a CX3C motif." Nature **385**(6617): 640-644.
- Beersma, M. F., M. J. Bijlmakers and H. L. Ploegh (1993). "Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains." J Immunol **151**(9): 4455-4464.
- Belldgrun, A., C. L. Tso, T. Sakata, T. Duckett, M. J. Brunda, S. H. Barsky, J. Chai, R. Kaboo, R. S. Lavey, W. H. McBride and et al. (1993). "Human renal carcinoma line transfected with interleukin-2 and/or interferon alpha gene(s): implications for live cancer vaccines." J Natl Cancer Inst **85**(3): 207-216.
- Benson, D. M., Jr., C. E. Bakan, A. Mishra, C. C. Hofmeister, Y. Efebera, B. Becknell, R. A. Baiocchi, J. Zhang, J. Yu, M. K. Smith, C. N. Greenfield, P. Porcu, S. M. Devine, R. Rotem-Yehudar, G. Lozanski, J. C. Byrd and M. A. Caligiuri (2010). "The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody." Blood **116**(13): 2286-2294.
- Berger, R., R. Rotem-Yehudar, G. Slama, S. Landes, A. Kneller, M. Leiba, M. Koren-Michowitz, A. Shimoni and A. Nagler (2008). "Phase I safety and pharmacokinetic study of CT-011, a

- humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies." *Clin Cancer Res* **14**(10): 3044-3051.
- Biassoni, R. (2008). "Natural killer cell receptors." *Adv Exp Med Biol* **640**: 35-52.
- Biassoni, R. (2009). "Human natural killer receptors, co-receptors, and their ligands." *Curr Protoc Immunol* **Chapter 14**: Unit 14 10.
- Bird, J. M., R. G. Owen, S. D'Sa, J. A. Snowden, G. Pratt, J. Ashcroft, K. Yong, G. Cook, S. Feyler, F. Davies, G. Morgan, J. Cavenagh, E. Low and J. Behrens (2011). "Guidelines for the diagnosis and management of multiple myeloma 2011." *Br J Haematol* **154**(1): 32-75.
- Bloushtain, N., U. Qimron, A. Bar-Ilan, O. HersHKovitz, R. Gazit, E. Fima, M. Korc, I. Vlodavsky, N. V. Bovin and A. Porgador (2004). "Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46." *J Immunol* **173**(4): 2392-2401.
- Borrego, F., M. J. Robertson, J. Ritz, J. Pena and R. Solana (1999). "CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor." *Immunology* **97**(1): 159-165.
- Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, M. Vitale, L. Moretta, M. Lopez and A. Moretta (2003). "Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule." *J Exp Med* **198**(4): 557-567.
- Boyman, O. and J. Sprent (2012). "The role of interleukin-2 during homeostasis and activation of the immune system." *Nat Rev Immunol* **12**(3): 180-190.
- Braga, W. M., D. Atanackovic and G. W. Colleoni (2012). "The role of regulatory T cells and TH17 cells in multiple myeloma." *Clin Dev Immunol* **2012**: 293479.
- Brill, T. H., H. R. Kubler, H. Pohla, A. Buchner, F. Fend, T. Schuster, H. van Randenborgh, R. Paul, T. Kummer, C. Plank, B. Eisele, J. Breul, R. Hartung, D. J. Schendel and B. Gansbacher (2009). "Therapeutic vaccination with an interleukin-2-interferon-gamma-secreting allogeneic tumor vaccine in patients with progressive castration-resistant prostate cancer: a phase I/II trial." *Hum Gene Ther* **20**(12): 1641-1651.
- Brilot, F., T. Strowig and C. Munz (2008). "NK cells interactions with dendritic cells shape innate and adaptive immunity." *Front Biosci* **13**: 6443-6454.
- Brown, R. D., B. Pope, A. Murray, W. Esdale, D. M. Sze, J. Gibson, P. J. Ho, D. Hart and D. Joshua (2001). "Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10." *Blood* **98**(10): 2992-2998.
- Brown, R. D., E. Yuen, M. Nelson, J. Gibson and D. Joshua (1997). "The prognostic significance of T cell receptor beta gene rearrangements and idiotype-reactive T cells in multiple myeloma." *Leukemia* **11**(8): 1312-1317.
- Bryceson, Y. T., S. C. Chiang, S. Darmanin, C. Fauriat, H. Schlums, J. Theorell and S. M. Wood (2011). "Molecular mechanisms of natural killer cell activation." *J Innate Immun* **3**(3): 216-226.
- Bryceson, Y. T., M. E. March, H. G. Ljunggren and E. O. Long (2006). "Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion." *Blood* **107**(1): 159-166.
- Bubenik, J., P. Rossner, D. Bubenikova, J. Simova, M. Indrova and E. Sloncova (1997). "Tumour vaccines expressing IL-2, CD80, and IL-2 plus CD80 gene." *Int J Oncol* **11**(6): 1213-1219.
- Buchner, A., H. Pohla, G. Willmsky, B. Frankenberger, R. Frank, A. Baur-Melnyk, M. Siebels, C. G. Stief, A. Hofstetter, J. Kopp, A. Pezzutto, T. Blankenstein, R. Oberneder and D. J. Schendel (2010). "Phase 1 trial of allogeneic gene-modified tumor cell vaccine RCC-26/CD80/IL-2 in patients with metastatic renal cell carcinoma." *Hum Gene Ther* **21**(3): 285-297.
- Burgess, S. J., K. Maasho, M. Masilamani, S. Narayanan, F. Borrego and J. E. Coligan (2008). "The NKG2D receptor: immunobiology and clinical implications." *Immunol Res* **40**(1): 18-34.
- Burgess, S. J., A. I. Marusina, I. Pathmanathan, F. Borrego and J. E. Coligan (2006). "IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells." *J Immunol* **176**(3): 1490-1497.
- Caligaris-Cappio, F., M. G. Gregoretti, F. Merico, D. Gottardi, P. Ghia, G. Parvis and L. Bergui (1992). "Bone marrow microenvironment and the progression of multiple myeloma." *Leuk Lymphoma* **8**(1-2): 15-22.

- Campbell, J. D., G. Cook, S. E. Robertson, A. Fraser, K. S. Boyd, J. A. Gracie and I. M. Franklin (2001a). "Suppression of IL-2-induced T cell proliferation and phosphorylation of STAT3 and STAT5 by tumor-derived TGF beta is reversed by IL-15." *J Immunol* **167**(1): 553-561.
- Campbell, J. J., S. Qin, D. Unutmaz, D. Soler, K. E. Murphy, M. R. Hodge, L. Wu and E. C. Butcher (2001b). "Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire." *J Immunol* **166**(11): 6477-6482.
- Cantoni, C., C. Bottino, M. Vitale, A. Pessino, R. Augugliaro, A. Malaspina, S. Parolini, L. Moretta, A. Moretta and R. Biassoni (1999). "NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily." *J Exp Med* **189**(5): 787-796.
- Carbone, E., P. Neri, M. Mesuraca, M. T. Fulciniti, T. Otsuki, D. Pende, V. Groh, T. Spies, G. Pollio, D. Cosman, L. Catalano, P. Tassone, B. Rotoli and S. Venuta (2005). "HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells." *Blood* **105**(1): 251-258.
- Carlos, C. A., H. F. Dong, O. M. Howard, J. J. Oppenheim, F. G. Hanisch and O. J. Finn (2005). "Human tumor antigen MUC1 is chemotactic for immature dendritic cells and elicits maturation but does not promote Th1 type immunity." *J Immunol* **175**(3): 1628-1635.
- Carpenter, E. L. and R. H. Vonderheide (2006). "Telomerase-based immunotherapy of cancer." *Expert Opin Biol Ther* **6**(10): 1031-1039.
- Carrega, P., G. Pezzino, P. Queirolo, I. Bonaccorsi, M. Falco, G. Vita, D. Pende, A. Misefari, A. Moretta, M. C. Mingari, L. Moretta and G. Ferlazzo (2009). "Susceptibility of human melanoma cells to autologous natural killer (NK) cell killing: HLA-related effector mechanisms and role of unlicensed NK cells." *PLoS One* **4**(12): e8132.
- Castriconi, R., C. Cantoni, M. Della Chiesa, M. Vitale, E. Marcenaro, R. Conte, R. Biassoni, C. Bottino, L. Moretta and A. Moretta (2003). "Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells." *Proc Natl Acad Sci U S A* **100**(7): 4120-4125.
- Cerwenka, A., J. L. Baron and L. L. Lanier (2001). "Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo." *Proc Natl Acad Sci U S A* **98**(20): 11521-11526.
- Chambers, B. J., M. Salcedo and H. G. Ljunggren (1996). "Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1)." *Immunity* **5**(4): 311-317.
- Chan, K. S. and A. Kaur (2007). "Flow cytometric detection of degranulation reveals phenotypic heterogeneity of degranulating CMV-specific CD8+ T lymphocytes in rhesus macaques." *J Immunol Methods* **325**(1-2): 20-34.
- Chan, L., N. Hardwick, D. Darling, J. Galea-Lauri, J. Gaken, S. Devereux, M. Kemeny, G. Mufti and F. Farzaneh (2005a). "IL-2/B7.1 (CD80) fusogene transduction of AML blasts by a self-inactivating lentiviral vector stimulates T cell responses in vitro: a strategy to generate whole cell vaccines for AML." *Mol Ther* **11**(1): 120-131.
- Chan, L., N. R. Hardwick, B. A. Guinn, D. Darling, J. Gaken, J. Galea-Lauri, A. Y. Ho, G. J. Mufti and F. Farzaneh (2006). "An immune edited tumour versus a tumour edited immune system: Prospects for immune therapy of acute myeloid leukaemia." *Cancer Immunol Immunother* **55**(8): 1017-1024.
- Chan, L., D. Nesbeth, T. Mackey, J. Galea-Lauri, J. Gaken, F. Martin, M. Collins, G. Mufti, F. Farzaneh and D. Darling (2005b). "Conjugation of lentivirus to paramagnetic particles via nonviral proteins allows efficient concentration and infection of primary acute myeloid leukemia cells." *J Virol* **79**(20): 13190-13194.
- Cignetti, A., A. Vallario, A. Follenzi, P. Circosta, A. Capaldi, D. Gottardi, L. Naldini and F. Caligaris-Cappio (2005). "Lentiviral transduction of primary myeloma cells with CD80 and CD154 generates antimyeloma effector T cells." *Hum Gene Ther* **16**(4): 445-456.
- Cooper, M. A., J. M. Elliott, P. A. Keyel, L. Yang, J. A. Carrero and W. M. Yokoyama (2009). "Cytokine-induced memory-like natural killer cells." *Proc Natl Acad Sci U S A* **106**(6): 1915-1919.
- Cooper, M. A., T. A. Fehniger and M. A. Caligiuri (2001). "The biology of human natural killer-cell subsets." *Trends Immunol* **22**(11): 633-640.
- Cooper, M. A., T. A. Fehniger, A. Fuchs, M. Colonna and M. A. Caligiuri (2004). "NK cell and DC interactions." *Trends Immunol* **25**(1): 47-52.
- Coscia, M., S. Mariani, S. Battaglio, C. Di Bello, F. Fiore, M. Foglietta, A. Pileri, M. Boccadoro and M. Massaia (2004). "Long-term follow-up of idiotypic vaccination in human myeloma as a maintenance therapy after high-dose chemotherapy." *Leukemia* **18**(1): 139-145.

- Coudert, J. D., J. Zimmer, E. Tomasello, M. Cebecauer, M. Colonna, E. Vivier and W. Held (2005). "Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells." *Blood* **106**(5): 1711-1717.
- Crawley, C., M. Lalancette, R. Szydlo, M. Gilleece, K. Peggs, S. Mackinnon, G. Juliusson, L. Ahlberg, A. Nagler, A. Shimoni, A. Sureda, J. M. Boiron, H. Einsele, R. Chopra, A. Carella, J. Cavenagh, A. Gratwohl, F. Garban, A. Zander, B. Bjorkstrand, D. Niederwieser, G. Gahrton and J. F. Apperley (2005). "Outcomes for reduced-intensity allogeneic transplantation for multiple myeloma: an analysis of prognostic factors from the Chronic Leukaemia Working Party of the EBMT." *Blood* **105**(11): 4532-4539.
- Danylesko, I., K. Beider, A. Shimoni and A. Nagler (2012). "Novel strategies for immunotherapy in multiple myeloma: previous experience and future directions." *Clin Dev Immunol* **2012**(9): 753407.
- Das, G., S. Sheridan and C. A. Janeway, Jr. (2001). "The source of early IFN-gamma that plays a role in Th1 priming." *J Immunol* **167**(4): 2004-2010.
- Davies, F. E., N. Raje, T. Hideshima, S. Lentzsch, G. Young, Y. T. Tai, B. Lin, K. Podar, D. Gupta, D. Chauhan, S. P. Treon, P. G. Richardson, R. L. Schlossman, G. J. Morgan, G. W. Muller, D. I. Stirling and K. C. Anderson (2001). "Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma." *Blood* **98**(1): 210-216.
- de Gruijl, T. D., A. J. van den Eertwegh, H. M. Pinedo and R. J. Scheper (2008). "Whole-cell cancer vaccination: from autologous to allogeneic tumor- and dendritic cell-based vaccines." *Cancer Immunol Immunother* **57**(10): 1569-1577.
- de Rham, C., S. Ferrari-Lacraz, S. Jendly, G. Schneiter, J. M. Dayer and J. Villard (2007). "The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors." *Arthritis Res Ther* **9**(6): R125.
- Degli-Esposti, M. A. and M. J. Smyth (2005). "Close encounters of different kinds: dendritic cells and NK cells take centre stage." *Nat Rev Immunol* **5**(2): 112-124.
- Della Chiesa, M., M. Vitale, S. Carlomagno, G. Ferlazzo, L. Moretta and A. Moretta (2003). "The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors." *Eur J Immunol* **33**(6): 1657-1666.
- Diefenbach, A., A. M. Jamieson, S. D. Liu, N. Shastri and D. H. Raulet (2000). "Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages." *Nat Immunol* **1**(2): 119-126.
- Diehl, S. and M. Rincon (2002). "The two faces of IL-6 on Th1/Th2 differentiation." *Mol Immunol* **39**(9): 531-536.
- Dols, A., J. W. Smith, 2nd, S. L. Meijer, B. A. Fox, H. M. Hu, E. Walker, S. Rosenheim, T. Moudgil, T. Doran, W. Wood, M. Seligman, W. G. Alvord, D. Schoof and W. J. Urba (2003). "Vaccination of women with metastatic breast cancer, using a costimulatory gene (CD80)-modified, HLA-A2-matched, allogeneic, breast cancer cell line: clinical and immunological results." *Hum Gene Ther* **14**(11): 1117-1123.
- Dredge, K., J. B. Marriott, S. M. Todryk, G. W. Muller, R. Chen, D. I. Stirling and A. G. Dalgleish (2002). "Protective antitumor immunity induced by a costimulatory thalidomide analog in conjunction with whole tumor cell vaccination is mediated by increased Th1-type immunity." *J Immunol* **168**(10): 4914-4919.
- Drexler, H. G. and Y. Matsuo (2000). "Malignant hematopoietic cell lines: in vitro models for the study of multiple myeloma and plasma cell leukemia." *Leuk Res* **24**(8): 681-703.
- Dropulic, B. (2011). "Lentiviral vectors: their molecular design, safety, and use in laboratory and preclinical research." *Hum Gene Ther* **22**(6): 649-657.
- DuBridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller and M. P. Calos (1987). "Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system." *Mol Cell Biol* **7**(1): 379-387.
- Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono and L. Naldini (1998). "A third-generation lentivirus vector with a conditional packaging system." *J Virol* **72**(11): 8463-8471.
- Dummer, R., F. Y. Yue, J. Pavlovic, R. Geertsen, C. Dohring, K. Moelling and G. Burg (1998). "Immune stimulatory potential of B7.1 and B7.2 retrovirally transduced melanoma cells: suppression by interleukin 10." *Br J Cancer* **77**(9): 1413-1419.
- Dunussi-Joannopoulos, K., G. Dranoff, H. J. Weinstein, J. L. Ferrara, B. E. Bierer and J. M. Croop (1998). "Gene immunotherapy in murine acute myeloid leukemia: granulocyte-macrophage colony-stimulating factor tumor cell vaccines elicit more potent antitumor immunity compared with B7 family and other cytokine vaccines." *Blood* **91**(1): 222-230.

- Dzierzak, E. (1999). "Embryonic beginnings of definitive hematopoietic stem cells." *Ann N Y Acad Sci* **872**: 256-262; discussion 262-254.
- Eagle, R. A. and J. Trowsdale (2007). "Promiscuity and the single receptor: NKG2D." *Nat Rev Immunol* **7**(9): 737-744.
- El-Sherbiny, Y. M., J. L. Meade, T. D. Holmes, D. McGonagle, S. L. Mackie, A. W. Morgan, G. Cook, S. Feyler, S. J. Richards, F. E. Davies, G. J. Morgan and G. P. Cook (2007). "The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells." *Cancer Res* **67**(18): 8444-8449.
- Elboim, M., R. Gazit, C. Gur, H. Ghadially, G. Betser-Cohen and O. Mandelboim (2010). "Tumor immunoediting by NKp46." *J Immunol* **184**(10): 5637-5644.
- Ferlazzo, G., M. Pack, D. Thomas, C. Paludan, D. Schmid, T. Strowig, G. Bougras, W. A. Muller, L. Moretta and C. Munz (2004). "Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs." *Proc Natl Acad Sci U S A* **101**(47): 16606-16611.
- Ferlazzo, G., M. L. Tsang, L. Moretta, G. Melioli, R. M. Steinman and C. Munz (2002). "Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells." *J Exp Med* **195**(3): 343-351.
- Fernandez, N. C., A. Lozier, C. Flament, P. Ricciardi-Castagnoli, D. Bellet, M. Suter, M. Perricaudet, T. Tursz, E. Maraskovsky and L. Zitvogel (1999). "Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo." *Nat Med* **5**(4): 405-411.
- Feyler, S., G. B. Scott, C. Parrish, S. Jarmin, P. Evans, M. Short, K. McKinley, P. J. Selby and G. Cook (2012). "Tumour cell generation of inducible regulatory T-cells in multiple myeloma is contact-dependent and antigen-presenting cell-independent." *PLoS One* **7**(5): e35981.
- Feyler, S., P. J. Selby and G. Cook (2013). "Regulating the regulators in cancer-immunosuppression in multiple myeloma (MM)." *Blood Rev* **27**(3): 155-164.
- Follenzi, A., G. Sabatino, A. Lombardo, C. Boccaccio and L. Naldini (2002). "Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors." *Hum Gene Ther* **13**(2): 243-260.
- Fonseca, R., P. L. Bergsagel, J. Drach, J. Shaughnessy, N. Gutierrez, A. K. Stewart, G. Morgan, B. Van Ness, M. Chesi, S. Minvielle, A. Neri, B. Barlogie, W. M. Kuehl, P. Liebisch, F. Davies, S. Chen-Kiang, B. G. Durie, R. Carrasco, O. Sezer, T. Reiman, L. Pilarski and H. Avet-Loiseau (2009). "International Myeloma Working Group molecular classification of multiple myeloma: spotlight review." *Leukemia* **23**(12): 2210-2221.
- Frankenberger, B., H. Pohla, E. Noessner, G. Willmsky, B. Papier, A. Pezzutto, J. Kopp, R. Oberneder, T. Blankenstein and D. J. Schendel (2005). "Influence of CD80, interleukin-2, and interleukin-7 expression in human renal cell carcinoma on the expansion, function, and survival of tumor-specific CTLs." *Clin Cancer Res* **11**(5): 1733-1742.
- Frohn, C., M. Hoppner, P. Schlenke, H. Kirchner, P. Koritke and J. Luhm (2002). "Anti-myeloma activity of natural killer lymphocytes." *Br J Haematol* **119**(3): 660-664.
- Gaken, J., J. Jiang, K. Daniel, E. van Berkel, C. Hughes, M. Kuiper, D. Darling, M. Tavassoli, J. Galea-Lauri, K. Ford, M. Kemeny, S. Russell and F. Farzaneh (2000). "Fusogene vectors: a novel strategy for the expression of multiple genes from a single cistron." *Gene Ther* **7**(23): 1979-1985.
- Galea-Lauri, J., D. Darling, S. U. Gan, L. Krivochtchapov, M. Kuiper, J. Gaken, B. Souberbielle and F. Farzaneh (1999). "Expression of a variant of CD28 on a subpopulation of human NK cells: implications for B7-mediated stimulation of NK cells." *J Immunol* **163**(1): 62-70.
- Garcia-Iglesias, T., A. Del Toro-Arreola, B. Albarran-Somoza, S. Del Toro-Arreola, P. E. Sanchez-Hernandez, M. G. Ramirez-Duenas, L. M. Balderas-Pena, A. Bravo-Cuellar, P. C. Ortiz-Lazareno and A. Daneri-Navarro (2009). "Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions." *BMC Cancer* **9**: 186.
- Geldhof, A. B., G. Raes, M. Bakkus, S. Devos, K. Thielemans and P. De Baetselier (1995). "Expression of B7-1 by highly metastatic mouse T lymphomas induces optimal natural killer cell-mediated cytotoxicity." *Cancer Res* **55**(13): 2730-2733.
- Ghio, M., P. Contini, S. Negrini, S. Boero, A. Musso and A. Poggi (2009). "Soluble HLA-I-mediated secretion of TGF-beta1 by human NK cells and consequent down-regulation of anti-tumor cytolytic activity." *Eur J Immunol* **39**(12): 3459-3468.
- Gialeli, C., A. D. Theocharis and N. K. Karamanos (2011). "Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting." *FEBS J* **278**(1): 16-27.
- Girlanda, S., C. Fortis, D. Belloni, E. Ferrero, P. Ticozzi, C. Sciorati, M. Tresoldi, A. Vicari, T. Spies, V. Groh, F. Caligaris-Cappio and M. Ferrarini (2005). "MICA expressed by multiple myeloma and monoclonal gammopathy of undetermined significance plasma cells

- Costimulates pamidronate-activated gammadelta lymphocytes." *Cancer Res* **65**(16): 7502-7508.
- Godfrey, J. and D. M. Benson, Jr. (2012). "The role of natural killer cells in immunity against multiple myeloma." *Leuk Lymphoma* **53**(9): 1666-1676.
- Gonzalez, D., M. van der Burg, R. Garcia-Sanz, J. A. Fenton, A. W. Langerak, M. Gonzalez, J. J. van Dongen, J. F. San Miguel and G. J. Morgan (2007). "Immunoglobulin gene rearrangements and the pathogenesis of multiple myeloma." *Blood* **110**(9): 3112-3121.
- Goodier, M. R. and M. Londei (2004). "CD28 is not directly involved in the response of human CD3- CD56+ natural killer cells to lipopolysaccharide: a role for T cells." *Immunology* **111**(4): 384-390.
- Groh, V., J. Wu, C. Yee and T. Spies (2002). "Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation." *Nature* **419**(6908): 734-738.
- Guckel, B., M. Lindauer, W. Rudy, A. Habicht, M. Siebels, S. Kaul, G. Bastert, S. C. Meuer and U. Moebius (1995). "CD80-transfected human breast and ovarian tumor cell lines: improved immunogenicity and induction of cytolytic CD8+ T lymphocytes." *Cytokines Mol Ther* **1**(3): 211-221.
- Guckel, B., G. C. Meyer, W. Rudy, R. Batrla, S. C. Meuer, G. Bastert, D. Wallwiener and U. Moebius (1999). "Interleukin-12 requires initial CD80-mediated T-cell activation to support immune responses toward human breast and ovarian carcinoma." *Cancer Gene Ther* **6**(3): 228-237.
- Guerra, N., Y. X. Tan, N. T. Joncker, A. Choy, F. Gallardo, N. Xiong, S. Knoblauth, D. Cado, N. M. Greenberg and D. H. Raulet (2008). "NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy." *Immunity* **28**(4): 571-580.
- Haanen, J. B., A. Baars, R. Gomez, P. Weder, M. Smits, T. D. de Gruijl, B. M. von Blomberg, E. Bloemena, R. J. Scheper, S. M. van Ham, H. M. Pinedo and A. J. van den Eertwegh (2006). "Melanoma-specific tumor-infiltrating lymphocytes but not circulating melanoma-specific T cells may predict survival in resected advanced-stage melanoma patients." *Cancer Immunol Immunother* **55**(4): 451-458.
- Haile, S. T., J. J. Bosch, N. I. Agu, A. M. Zeender, P. Somasundaram, M. K. Srivastava, S. Britting, J. B. Wolf, B. R. Ksander and S. Ostrand-Rosenberg (2011). "Tumor cell programmed death ligand 1-mediated T cell suppression is overcome by coexpression of CD80." *J Immunol* **186**(12): 6822-6829.
- Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." *J Mol Biol* **166**(4): 557-580.
- Hanna, M. G., Jr. and L. C. Peters (1978). "Immunotherapy of established micrometastases with Bacillus Calmette-Guerin tumor cell vaccine." *Cancer Res* **38**(1): 204-209.
- Hardwick, N., L. Chan, W. Ingram, G. Mufti and F. Farzaneh (2010). "Lytic activity against primary AML cells is stimulated in vitro by an autologous whole cell vaccine expressing IL-2 and CD80." *Cancer Immunol Immunother* **59**(3): 379-388.
- Harrison, S. J. and G. Cook (2005). "Immunotherapy in multiple myeloma--possibility or probability?" *Br J Haematol* **130**(3): 344-362.
- Hayashi, T., T. Hideshima, M. Akiyama, K. Podar, H. Yasui, N. Raje, S. Kumar, D. Chauhan, S. P. Treon, P. Richardson and K. C. Anderson (2005). "Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application." *Br J Haematol* **128**(2): 192-203.
- Herberman, R. B., M. E. Nunn, H. T. Holden and D. H. Lavrin (1975). "Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells." *Int J Cancer* **16**(2): 230-239.
- Hicks, C., C. Cheung and R. Lindeman (2003). "Restimulation of tumour-specific immunity in a patient with AML following injection with B7-1 positive autologous blasts." *Leuk Res* **27**(11): 1051-1061.
- Hideshima, T., C. Mitsiades, G. Tonon, P. G. Richardson and K. C. Anderson (2007). "Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets." *Nat Rev Cancer* **7**(8): 585-598.
- Hill, A. B., B. C. Barnett, A. J. McMichael and D. J. McGeoch (1994). "HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2." *J Immunol* **152**(6): 2736-2741.
- Hirst, W. J., A. Buggins, D. Darling, J. Gaken, F. Farzaneh and G. J. Mufti (1997). "Enhanced immune costimulatory activity of primary acute myeloid leukaemia blasts after retrovirus-mediated gene transfer of B7.1." *Gene Ther* **4**(7): 691-699.
- Horst, A., N. Hunzelmann, S. Arce, M. Herber, R. A. Manz, A. Radbruch, R. Nischt, J. Schmitz and M. Assenmacher (2002). "Detection and characterization of plasma cells in peripheral

- blood: correlation of IgE+ plasma cell frequency with IgE serum titre." Clin Exp Immunol **130**(3): 370-378.
- Hsu, A. K., H. Quach, T. Tai, H. M. Prince, S. J. Harrison, J. A. Trapani, M. J. Smyth, P. Neeson and D. S. Ritchie (2011). "The immunostimulatory effect of lenalidomide on NK-cell function is profoundly inhibited by concurrent dexamethasone therapy." Blood **117**(5): 1605-1613.
- Hu, B., A. Tai and P. Wang (2011). "Immunization delivered by lentiviral vectors for cancer and infectious diseases." Immunol Rev **239**(1): 45-61.
- Iguchi-Manaka, A., H. Kai, Y. Yamashita, K. Shibata, S. Tahara-Hanaoka, S. Honda, T. Yasui, H. Kikutani, K. Shibuya and A. Shibuya (2008). "Accelerated tumor growth in mice deficient in DNAM-1 receptor." J Exp Med **205**(13): 2959-2964.
- Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiya, T. J. Schall and O. Yoshie (1997). "Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion." Cell **91**(4): 521-530.
- Ingram, W., L. Chan, H. Guven, D. Darling, S. Kordasti, N. Hardwick, L. Barber, G. J. Mufti and F. Farzaneh (2009). "Human CD80/IL2 lentivirus-transduced acute myeloid leukaemia (AML) cells promote natural killer (NK) cell activation and cytolytic activity: implications for a phase I clinical study." Br J Haematol **145**(6): 749-760.
- Joshua, D. E., R. D. Brown, P. J. Ho and J. Gibson (2008). "Regulatory T cells and multiple myeloma." Clin Lymphoma Myeloma **8**(5): 283-286.
- Juriscic, V., T. Srdic, G. Konjevic, O. Markovic and M. Colovic (2007). "Clinical stage-depending decrease of NK cell activity in multiple myeloma patients." Med Oncol **24**(3): 312-317.
- Kalinski, P., A. Giermasz, Y. Nakamura, P. Basse, W. J. Storkus, J. M. Kirkwood and R. B. Mailliard (2005). "Helper role of NK cells during the induction of anticancer responses by dendritic cells." Mol Immunol **42**(4): 535-539.
- Katodritou, E., E. Terpos, J. North, P. Kottaridis, E. Verrou, V. Gastari, C. Chadjiaggelidou, S. Sivakumaran, S. Jide-Banwo, M. Tsirogianni, D. Kapetanios, K. Zervas and M. W. Lowdell (2011). "Tumor-primed natural killer cells from patients with multiple myeloma lyse autologous, NK-resistant, bone marrow-derived malignant plasma cells." Am J Hematol **86**(12): 967-973.
- Kaufmann, A. M., L. Gissmann, P. Simms, C. Schreckenberger and L. Qiao (2000). "Comparison of cytokines and CD80 for enhancement of immunogenicity of cervical cancer cells." Immunobiology **202**(4): 339-352.
- Kloss, M., P. Decker, K. M. Baltz, T. Baessler, G. Jung, H. G. Rammensee, A. Steinle, M. Krusch and H. R. Salih (2008). "Interaction of monocytes with NK cells upon Toll-like receptor-induced expression of the NKG2D ligand MICA." J Immunol **181**(10): 6711-6719.
- Koeffler, H. P. and D. W. Golde (1980). "Human myeloid leukemia cell lines: a review." Blood **56**(3): 344-350.
- Korinek, V., I. Stefanova, P. Angelisova, I. Hilgert and V. Horejsi (1991). "The human leucocyte antigen CD48 (MEM-102) is closely related to the activation marker Blast-1." Immunogenetics **33**(2): 108-112.
- Koya, R. C., N. Kasahara, V. Pullarkat, A. M. Levine and R. Stripecke (2002). "Transduction of acute myeloid leukemia cells with third generation self-inactivating lentiviral vectors expressing CD80 and GM-CSF: effects on proliferation, differentiation, and stimulation of allogeneic and autologous anti-leukemia immune responses." Leukemia **16**(9): 1645-1654.
- Kyle, R. A. and S. V. Rajkumar (2003). "Monoclonal gammopathies of undetermined significance: a review." Immunol Rev **194**: 112-139.
- Kyle, R. A. and S. V. Rajkumar (2007). "Monoclonal gammopathy of undetermined significance and smoldering multiple myeloma: emphasis on risk factors for progression." Br J Haematol **139**(5): 730-743.
- Lanier, L. L. (2005). "NK cell recognition." Annu Rev Immunol **23**: 225-274.
- LeBlanc, R., T. Hideshima, L. P. Catley, R. Shringarpure, R. Burger, N. Mitsiades, C. Mitsiades, P. Cheema, D. Chauhan, P. G. Richardson, K. C. Anderson and N. C. Munshi (2004). "Immunomodulatory drug costimulates T cells via the B7-CD28 pathway." Blood **103**(5): 1787-1790.
- Leibson, P. J. (1997). "Signal transduction during natural killer cell activation: inside the mind of a killer." Immunity **6**(6): 655-661.
- Li, C., B. Ge, M. Nicotra, J. N. Stern, H. D. Kopcow, X. Chen and J. L. Strominger (2008). "JNK MAP kinase activation is required for MTOC and granule polarization in NKG2D-mediated NK cell cytotoxicity." Proc Natl Acad Sci U S A **105**(8): 3017-3022.

- Lim, S. H., Z. Wang, M. Chiriva-Internati and Y. Xue (2001). "Sperm protein 17 is a novel cancer-testis antigen in multiple myeloma." Blood **97**(5): 1508-1510.
- Liu, J., A. Hamrouni, D. Wolowiec, V. Coiteux, K. Kuliczowski, D. Hetuin, A. Saudemont and B. Quesnel (2007). "Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN- γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway." Blood **110**(1): 296-304.
- Ljunggren, H. G. and K. Karre (1986). "Experimental strategies and interpretations in the analysis of changes in MHC gene expression during tumour progression. Opposing influences of T cell and natural killer mediated resistance?" J Immunogenet **13**(2-3): 141-151.
- Ljunggren, H. G. and K. Karre (1990). "In search of the 'missing self': MHC molecules and NK cell recognition." Immunol Today **11**(7): 237-244.
- Long, E. O. (2008). "Negative signaling by inhibitory receptors: the NK cell paradigm." Immunol Rev **224**: 70-84.
- Lotze, M. T., E. A. Grimm, A. Mazumder, J. L. Strausser and S. A. Rosenberg (1981). "Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor." Cancer Res **41**(11 Pt 1): 4420-4425.
- Lotze, M. T. and S. A. Rosenberg (1986). "Results of clinical trials with the administration of interleukin 2 and adoptive immunotherapy with activated cells in patients with cancer." Immunobiology **172**(3-5): 420-437.
- Louis, N., C. Eveleigh and F. L. Graham (1997). "Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line." Virology **233**(2): 423-429.
- Lu, Z. Y., M. Condomines, K. Tarte, L. Nadal, M. C. Delteil, J. F. Rossi, C. Ferrand and B. Klein (2007). "B7-1 and 4-1BB ligand expression on a myeloma cell line makes it possible to expand autologous tumor-specific cytotoxic T cells in vitro." Exp Hematol **35**(3): 443-453.
- Lucas, M., W. Schachterle, K. Oberle, P. Aichele and A. Diefenbach (2007). "Dendritic cells prime natural killer cells by trans-presenting interleukin 15." Immunity **26**(4): 503-517.
- Luptakova, K., J. Rosenblatt, B. Glotzbecker, H. Mills, D. Stroopinsky, T. Kufe, B. Vasir, J. Arnason, D. Tzachanis, J. I. Zwicker, R. M. Joyce, J. D. Levine, K. C. Anderson, D. Kufe and D. Avigan (2013). "Lenalidomide enhances anti-myeloma cellular immunity." Cancer Immunol Immunother **62**(1): 39-49.
- Luque, I., H. Reyburn and J. L. Strominger (2000). "Expression of the CD80 and CD86 molecules enhances cytotoxicity by human natural killer cells." Hum Immunol **61**(8): 721-728.
- Mackey, M. F., R. J. Barth, Jr. and R. J. Noelle (1998). "The role of CD40/CD154 interactions in the priming, differentiation, and effector function of helper and cytotoxic T cells." J Leukoc Biol **63**(4): 418-428.
- Mahnke, Y. D. and M. Roederer (2007). "Optimizing a multicolor immunophenotyping assay." Clin Lab Med **27**(3): 469-485, v.
- Maki, G., G. M. Hayes, A. Naji, T. Tyler, E. D. Carosella, N. Rouas-Freiss and S. A. Gregory (2008). "NK resistance of tumor cells from multiple myeloma and chronic lymphocytic leukemia patients: implication of HLA-G." Leukemia **22**(5): 998-1006.
- Maloney, D. G., A. J. Molina, F. Sahebi, K. E. Stockerl-Goldstein, B. M. Sandmaier, W. Bensinger, B. Storer, U. Hegenbart, G. Somlo, T. Chauncey, B. Bruno, F. R. Appelbaum, K. G. Blume, S. J. Forman, P. McSweeney and R. Storb (2003). "Allografting with nonmyeloablative conditioning following cytoreductive autografts for the treatment of patients with multiple myeloma." Blood **102**(9): 3447-3454.
- Mamessier, E., A. Sylvain, F. Bertucci, R. Castellano, P. Finetti, G. Houvenaeghel, E. Charaffe-Jaufret, D. Birnbaum, A. Moretta and D. Olive (2011). "Human breast tumor cells induce self-tolerance mechanisms to avoid NKG2D-mediated and DNAM-mediated NK cell recognition." Cancer Res **71**(21): 6621-6632.
- Manickasingham, S. P., S. M. Anderton, C. Burkhardt and D. C. Wraith (1998). "Qualitative and quantitative effects of CD28/B7-mediated costimulation on naive T cells in vitro." J Immunol **161**(8): 3827-3835.
- Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia and F. Sallusto (2004). "Induced recruitment of NK cells to lymph nodes provides IFN- γ for T(H)1 priming." Nat Immunol **5**(12): 1260-1265.
- Matsuzaki, H., T. Kagimoto, T. Oda, F. Kawano and K. Takatsuki (1985). "Natural killer activity and antibody-dependent cell-mediated cytotoxicity in multiple myeloma." Jpn J Clin Oncol **15**(4): 611-617.
- Mitsiades, C. S., N. S. Mitsiades, N. C. Munshi, P. G. Richardson and K. C. Anderson (2006). "The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: interplay of growth factors, their receptors and stromal interactions." Eur J Cancer **42**(11): 1564-1573.

- Mollenhauer, H. H., D. J. Morre and L. D. Rowe (1990). "Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity." Biochim Biophys Acta **1031**(2): 225-246.
- Monti, P., B. E. Leone, A. Zerbi, G. Balzano, S. Cainarca, V. Sordi, M. Pontillo, A. Mercalli, V. Di Carlo, P. Allavena and L. Piemonti (2004). "Tumor-derived MUC1 mucins interact with differentiating monocytes and induce IL-10^{high}IL-12^{low} regulatory dendritic cell." J Immunol **172**(12): 7341-7349.
- Moretta, L. and A. Moretta (2004). "Unravelling natural killer cell function: triggering and inhibitory human NK receptors." EMBO J **23**(2): 255-259.
- Morgan, G. J., B. A. Walker and F. E. Davies (2012). "The genetic architecture of multiple myeloma." Nat Rev Cancer **12**(5): 335-348.
- Mota, G., I. Moldovan, A. Calugaru, M. Hirt, E. Kozma, C. Galatiuc, L. Brasoveanu and G. Boltz-Nitulescu (2004). "Interaction of human immunoglobulin G with CD16 on natural killer cells: ligand clearance, FcγRIIIA turnover and effects of metalloproteinases on FcγRIIIA-mediated binding, signal transduction and killing." Scand J Immunol **59**(3): 278-284.
- Mukherjee, S., D. Nelson, S. Loh, I. van Bruggen, L. J. Palmer, C. Leong, M. J. Garlepp and B. W. Robinson (2001). "The immune anti-tumor effects of GM-CSF and B7-1 gene transfection are enhanced by surgical debulking of tumor." Cancer Gene Ther **8**(8): 580-588.
- Mulders, P., C. L. Tso, S. Pang, R. Kaboo, W. H. McBride, A. Hinkel, B. Gitlitz, J. Dannull, R. Figlin and A. Belldegrun (1998). "Adenovirus-mediated interleukin-2 production by tumors induces growth of cytotoxic tumor-infiltrating lymphocytes against human renal cell carcinoma." J Immunother **21**(3): 170-180.
- Muntasell, A., G. Magri, D. Pende, A. Angulo and M. Lopez-Botet (2010). "Inhibition of NKG2D expression in NK cells by cytokines secreted in response to human cytomegalovirus infection." Blood **115**(25): 5170-5179.
- Murphy, K. (2011). Janeway's Immunobiology, Garland Science.
- Mutis, T., E. Schrama, C. J. Melief and E. Goulmy (1998). "CD80-Transfected acute myeloid leukemia cells induce primary allogeneic T-cell responses directed at patient specific minor histocompatibility antigens and leukemia-associated antigens." Blood **92**(5): 1677-1684.
- Naldini, L. (2009). "Medicine. A comeback for gene therapy." Science **326**(5954): 805-806.
- Nesbeth, D., S. L. Williams, L. Chan, T. Brain, N. K. Slater, F. Farzaneh and D. Darling (2006). "Metabolic biotinylation of lentiviral pseudotypes for scalable paramagnetic microparticle-dependent manipulation." Mol Ther **13**(4): 814-822.
- Nguyen-Pham, T. N., Y. K. Lee, H. J. Kim and J. J. Lee (2012). "Immunotherapy using dendritic cells against multiple myeloma: how to improve?" Clin Dev Immunol **2012**: 397648.
- Nguyen-Pham, T. N., M. S. Lim, T. A. Nguyen, Y. K. Lee, C. J. Jin, H. J. Lee, C. Y. Hong, J. S. Ahn, D. H. Yang, Y. K. Kim, I. J. Chung, B. C. Park, H. J. Kim and J. J. Lee (2011). "Type I and II interferons enhance dendritic cell maturation and migration capacity by regulating CD38 and CD74 that have synergistic effects with TLR agonists." Cell Mol Immunol **8**(4): 341-347.
- Nitta, T., H. Yagita, K. Sato and K. Okumura (1989). "Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction." J Exp Med **170**(5): 1757-1761.
- North, J., I. Bakhsh, C. Marden, H. Pittman, E. Addison, C. Navarrete, R. Anderson and M. W. Lowdell (2007). "Tumor-primed human natural killer cells lyse NK-resistant tumor targets: evidence of a two-stage process in resting NK cell activation." J Immunol **178**(1): 85-94.
- Obermayer, N., J. L. Wong, R. P. Edwards, K. Odunsi, K. Moysich and P. Kalinski (2012). "PGE(2)-driven induction and maintenance of cancer-associated myeloid-derived suppressor cells." Immunol Invest **41**(6-7): 635-657.
- Ogumundsdottir, H. M. (1988). "Natural killer cell activity in patients with multiple myeloma." Cancer Detect Prev **12**(1-6): 133-143.
- Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi and A. C. Hayday (2005). "Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance." Nat Immunol **6**(9): 928-937.
- Orsini, E., E. P. Alyea, R. Schlossman, C. Canning, R. J. Soiffer, A. Chillemi, D. Neuberg, K. C. Anderson and J. Ritz (2000). "Changes in T cell receptor repertoire associated with graft-versus-tumor effect and graft-versus-host disease in patients with relapsed multiple myeloma after donor lymphocyte infusion." Bone Marrow Transplant **25**(6): 623-632.
- Palumbo, A. and K. Anderson (2011). "Multiple myeloma." N Engl J Med **364**(11): 1046-1060.

- Parham, P. (2005). "MHC class I molecules and KIRs in human history, health and survival." Nat Rev Immunol **5**(3): 201-214.
- Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, C. Bottino, L. Moretta and A. Moretta (1999). "Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells." J Exp Med **190**(10): 1505-1516.
- Perez-Andres, M., J. Almeida, M. Martin-Ayuso, M. J. Moro, G. Martin-Nunez, J. Galende, D. Borrego, M. J. Rodriguez, F. Ortega, J. Hernandez, I. Moreno, M. Dominguez, G. Mateo, J. F. San Miguel and A. Orfao (2005). "Clonal plasma cells from monoclonal gammopathy of undetermined significance, multiple myeloma and plasma cell leukemia show different expression profiles of molecules involved in the interaction with the immunological bone marrow microenvironment." Leukemia **19**(3): 449-455.
- Pessino, A., S. Sivori, C. Bottino, A. Malaspina, L. Morelli, L. Moretta, R. Biassoni and A. Moretta (1998). "Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity." J Exp Med **188**(5): 953-960.
- Phekoo, K. J., S. A. Schey, M. A. Richards, D. H. Bevan, S. Bell, D. Gillett and H. Moller (2004). "A population study to define the incidence and survival of multiple myeloma in a National Health Service Region in UK." Br J Haematol **127**(3): 299-304.
- Pietra, G., C. Manzini, S. Rivara, M. Vitale, C. Cantoni, A. Petretto, M. Balsamo, R. Conte, R. Benelli, S. Minghelli, N. Solari, M. Gualco, P. Queirolo, L. Moretta and M. C. Mingari (2012). "Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity." Cancer Res **72**(6): 1407-1415.
- Platt, E. J., S. L. Kozak, J. P. Durnin, T. J. Hope and D. Kabat (2010). "Rapid dissociation of HIV-1 from cultured cells severely limits infectivity assays, causes the inactivation ascribed to entry inhibitors, and masks the inherently high level of infectivity of virions." J Virol **84**(6): 3106-3110.
- Podar, K., D. Chauhan and K. C. Anderson (2009). "Bone marrow microenvironment and the identification of new targets for myeloma therapy." Leukemia **23**(1): 10-24.
- Poggi, A., A. M. Massaro, S. Negrini, P. Contini and M. R. Zocchi (2005). "Tumor-induced apoptosis of human IL-2-activated NK cells: role of natural cytotoxicity receptors." J Immunol **174**(5): 2653-2660.
- Poggi, A. and M. R. Zocchi (2007). "Human natural killer lymphocytes through the engagement of natural cytotoxicity receptors and NKG2D can trigger self-aggression." Autoimmun Rev **6**(5): 295-299.
- Pratt, G., O. Goodyear and P. Moss (2007). "Immunodeficiency and immunotherapy in multiple myeloma." Br J Haematol **138**(5): 563-579.
- Quach, H., D. Ritchie, A. K. Stewart, P. Neeson, S. Harrison, M. J. Smyth and H. M. Prince (2010). "Mechanism of action of immunomodulatory drugs (IMiDs) in multiple myeloma." Leukemia **24**(1): 22-32.
- Raitakari, M., R. D. Brown, J. Gibson and D. E. Joshua (2003). "T cells in myeloma." Hematol Oncol **21**(1): 33-42.
- Rajkumar, S. V. (2011). "Treatment of multiple myeloma." Nat Rev Clin Oncol **8**(8): 479-491.
- Raulet, D. H. (2003). "Roles of the NKG2D immunoreceptor and its ligands." Nat Rev Immunol **3**(10): 781-790.
- Reichardt, V. L., C. Y. Okada, A. Liso, C. J. Benike, K. E. Stockerl-Goldstein, E. G. Engleman, K. G. Blume and R. Levy (1999). "Idiotypic vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma--a feasibility study." Blood **93**(7): 2411-2419.
- Ren, S. P., C. T. Wu, W. R. Huang, Z. Z. Lu, X. X. Jia, L. Wang, M. F. Lao and L. S. Wang (2006). "Adenoviral-mediated transfer of human wild-type p53, GM-CSF and B7-1 genes results in growth suppression and autologous anti-tumor cytotoxicity of multiple myeloma cells in vitro." Cancer Immunol Immunother **55**(4): 375-385.
- Rice, A. and D. Hart (2002). "Technology evaluation: APC-80200, Dendreon." Curr Opin Mol Ther **4**(5): 523-527.
- Robertson, M. J., R. J. Soiffer, S. F. Wolf, T. J. Manley, C. Donahue, D. Young, S. H. Herrmann and J. Ritz (1992). "Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF." J Exp Med **175**(3): 779-788.
- Roda-Navarro, P., M. Vales-Gomez, S. E. Chisholm and H. T. Reyburn (2006). "Transfer of NKG2D and MICB at the cytotoxic NK cell immune synapse correlates with a reduction in NK cell cytotoxic function." Proc Natl Acad Sci U S A **103**(30): 11258-11263.
- Rosenberg, S. A., M. T. Lotze, L. M. Muul, A. E. Chang, F. P. Avis, S. Leitman, W. M. Linehan, C. N. Robertson, R. E. Lee, J. T. Rubin and et al. (1987). "A progress report on the

- treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone." *N Engl J Med* **316**(15): 889-897.
- Rosenberg, S. A., M. T. Lotze, L. M. Muul, S. Leitman, A. E. Chang, S. E. Ettinghausen, Y. L. Matory, J. M. Skibber, E. Shiloni, J. T. Vetto and et al. (1985). "Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer." *N Engl J Med* **313**(23): 1485-1492.
- Rosenblatt, J. and D. Avigan (2008). "Cellular immunotherapy for multiple myeloma." *Best Pract Res Clin Haematol* **21**(3): 559-577.
- Rosenblatt, J., B. Vasir, L. Uhl, S. Blotta, C. Macnamara, P. Somaiya, Z. Wu, R. Joyce, J. D. Levine, D. Dombagoda, Y. E. Yuan, K. Francoeur, D. Fitzgerald, P. Richardson, E. Weller, K. Anderson, D. Kufe, N. Munshi and D. Avigan (2011). "Vaccination with dendritic cell/tumor fusion cells results in cellular and humoral antitumor immune responses in patients with multiple myeloma." *Blood* **117**(2): 393-402.
- Rousalova, I. and E. Krepela (2010). "Granzyme B-induced apoptosis in cancer cells and its regulation (review)." *Int J Oncol* **37**(6): 1361-1378.
- Rudy, W., B. Guckel, M. Siebels, M. Lindauer, S. C. Meuer and U. Moebius (1997). "Differential function of CD80- and CD86-transfected human melanoma cells in the presence of IL-12 and IFN-gamma." *Int Immunol* **9**(6): 853-860.
- Rugghetti, A., I. Pellicciotta, M. Biffoni, M. Backstrom, T. Link, E. P. Bennet, H. Clausen, T. Noll, G. C. Hansson, J. M. Burchell, L. Frati, J. Taylor-Papadimitriou and M. Nuti (2005). "Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells." *J Immunol* **174**(12): 7764-7772.
- Rutella, S. and F. Locatelli (2012). "Targeting multiple-myeloma-induced immune dysfunction to improve immunotherapy outcomes." *Clin Dev Immunol* **2012**: 196063.
- Sakuma, T., S. S. De Ravin, J. M. Tonne, T. Thatava, S. Ohmine, Y. Takeuchi, H. L. Malech and Y. Ikeda (2010). "Characterization of retroviral and lentiviral vectors pseudotyped with xenotropic murine leukemia virus-related virus envelope glycoprotein." *Hum Gene Ther* **21**(12): 1665-1673.
- Sanchez, C. J., T. Le Treut, A. Boehrer, B. Knoblauch, J. Imbert, D. Olive and R. T. Costello (2011). "Natural killer cells and malignant haemopathies: a model for the interaction of cancer with innate immunity." *Cancer Immunol Immunother* **60**(1): 1-13.
- Sandler, A. D., H. Chihara, G. Kobayashi, X. Zhu, M. A. Miller, D. L. Scott and A. M. Krieg (2003). "CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma." *Cancer Res* **63**(2): 394-399.
- Schendel, D. J., B. Frankenberger, P. Jantzer, S. Cayeux, E. Nobetaner, G. Willmsky, B. Maget, H. Pohla and T. Blankenstein (2000). "Expression of B7.1 (CD80) in a renal cell carcinoma line allows expansion of tumor-associated cytotoxic T lymphocytes in the presence of an alloresponse." *Gene Ther* **7**(23): 2007-2014.
- Schey, S. A., P. Fields, J. B. Bartlett, I. A. Clarke, G. Ashan, R. D. Knight, M. Streetly and A. G. Dalglish (2004). "Phase I study of an immunomodulatory thalidomide analog, CC-4047, in relapsed or refractory multiple myeloma." *J Clin Oncol* **22**(16): 3269-3276.
- Schmidt-Weber, C. B., J. G. Wohlfahrt, C. A. Akdis and K. Blaser (2002). "The phosphatidylinositol phosphatase PTEN is under control of costimulation and regulates proliferation in human T cells." *Eur J Immunol* **32**(4): 1196-1204.
- Seliger, B. (2008). "Molecular mechanisms of MHC class I abnormalities and APM components in human tumors." *Cancer Immunol Immunother* **57**(11): 1719-1726.
- Sharabi, A. and N. H. Ghera (2010). "Breaking tolerance in a mouse model of multiple myeloma by chemoimmunotherapy." *Adv Cancer Res* **107**: 1-37.
- Shibuya, K., L. L. Lanier, J. H. Phillips, H. D. Ochs, K. Shimizu, E. Nakayama, H. Nakauchi and A. Shibuya (1999). "Physical and functional association of LFA-1 with DNAM-1 adhesion molecule." *Immunity* **11**(5): 615-623.
- Sivori, S., D. Pende, C. Bottino, E. Marcenaro, A. Pessino, R. Biassoni, L. Moretta and A. Moretta (1999). "NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells." *Eur J Immunol* **29**(5): 1656-1666.
- Soriani, A., A. Zingoni, C. Cerboni, M. L. Iannitto, M. R. Ricciardi, V. Di Galleonardo, M. Cippitelli, C. Fionda, M. T. Petrucci, A. Guarini, R. Foa and A. Santoni (2009). "ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype." *Blood* **113**(15): 3503-3511.

- Spaggiari, G. M., A. Capobianco, H. Abdelrazik, F. Becchetti, M. C. Mingari and L. Moretta (2008). "Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2." Blood **111**(3): 1327-1333.
- Speiser, D. E., M. Migliaccio, M. J. Pittet, D. Valmori, D. Lienard, F. Lejeune, P. Reichenbach, P. Guillaume, I. Luscher, J. C. Cerottini and P. Romero (2001). "Human CD8(+) T cells expressing HLA-DR and CD28 show telomerase activity and are distinct from cytolytic effector T cells." Eur J Immunol **31**(2): 459-466.
- Strang, B. L., Y. Ikeda, F. L. Cosset, M. K. Collins and Y. Takeuchi (2004). "Characterization of HIV-1 vectors with gammaretrovirus envelope glycoproteins produced from stable packaging cells." Gene Ther **11**(7): 591-598.
- Stripecke, R., A. A. Cardoso, K. A. Pepper, D. C. Skelton, X. J. Yu, L. Mascarenhas, K. I. Weinberg, L. M. Nadler and D. B. Kohn (2000). "Lentiviral vectors for efficient delivery of CD80 and granulocyte-macrophage colony-stimulating factor in human acute lymphoblastic leukemia and acute myeloid leukemia cells to induce antileukemic immune responses." Blood **96**(4): 1317-1326.
- Stripecke, R., D. C. Skelton, T. Gruber, D. Afar, P. K. Pattengale, O. N. Witte and D. B. Kohn (1998). "Immune response to Philadelphia chromosome-positive acute lymphoblastic leukemia induced by expression of CD80, interleukin 2, and granulocyte-macrophage colony-stimulating factor." Hum Gene Ther **9**(14): 2049-2062.
- Stripecke, R., D. C. Skelton, P. K. Pattengale, H. Shimada and D. B. Kohn (1999). "Combination of CD80 and granulocyte-macrophage colony-stimulating factor coexpression by a leukemia cell vaccine: preclinical studies in a murine model recapitulating Philadelphia chromosome-positive acute lymphoblastic leukemia." Hum Gene Ther **10**(13): 2109-2122.
- Tani, K., M. Azuma, Y. Nakazaki, N. Oyaizu, H. Hase, J. Ohata, K. Takahashi, M. OiwaMonna, K. Hanazawa, Y. Wakumoto, K. Kawai, M. Noguchi, Y. Soda, R. Kunisaki, K. Watari, S. Takahashi, U. Machida, N. Satoh, A. Tojo, T. Maekawa, M. Eriguchi, S. Tomikawa, H. Tahara, Y. Inoue, H. Yoshikawa, Y. Yamada, A. Iwamoto, H. Hamada, N. Yamashita, K. Okumura, T. Kakizoe, H. Akaza, M. Fujime, S. Clift, D. Ando, R. Mulligan and S. Asano (2004). "Phase I study of autologous tumor vaccines transduced with the GM-CSF gene in four patients with stage IV renal cell cancer in Japan: clinical and immunological findings." Mol Ther **10**(4): 799-816.
- Tarte, K., X. G. Zhang, E. Legouffe, C. Hertog, M. Mehtali, J. F. Rossi and B. Klein (1999). "Induced expression of B7-1 on myeloma cells following retroviral gene transfer results in tumor-specific recognition by cytotoxic T cells." J Immunol **163**(1): 514-524.
- Terrazzano, G., D. Zanzi, C. Palomba, E. Carbone, S. Grimaldi, S. Pisanti, S. Fontana, S. Zappacosta and G. Ruggiero (2002). "Differential involvement of CD40, CD80, and major histocompatibility complex class I molecules in cytotoxicity induction and interferon-gamma production by human natural killer effectors." J Leukoc Biol **72**(2): 305-311.
- Valiante, N. M., K. Lienert, H. G. Shilling, B. J. Smits and P. Parham (1997). "Killer cell receptors: keeping pace with MHC class I evolution." Immunol Rev **155**: 155-164.
- van den Broeke, L. T., E. Daschbach, E. K. Thomas, G. Andringa and J. A. Berzofsky (2003). "Dendritic cell-induced activation of adaptive and innate antitumor immunity." J Immunol **171**(11): 5842-5852.
- van Kuppeveld, F. J., J. T. van der Logt, A. F. Angulo, M. J. van Zoest, W. G. Quint, H. G. Niesters, J. M. Galama and W. J. Melchers (1992). "Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification." Appl Environ Microbiol **58**(8): 2606-2615.
- Vasir, B., V. Borges, Z. Wu, D. Grosman, J. Rosenblatt, M. Irie, K. Anderson, D. Kufe and D. Avigan (2005). "Fusion of dendritic cells with multiple myeloma cells results in maturation and enhanced antigen presentation." Br J Haematol **129**(5): 687-700.
- Vereecque, R., G. Buffenoir, C. Preudhomme, D. Hetuin, F. Bauters, P. Fenaux and B. Quesnel (2000). "Gene transfer of GM-CSF, CD80 and CD154 cDNA enhances survival in a murine model of acute leukemia with persistence of a minimal residual disease." Gene Ther **7**(15): 1312-1316.
- Vignali, D. A., L. W. Collison and C. J. Workman (2008). "How regulatory T cells work." Nat Rev Immunol **8**(7): 523-532.
- Villunger, A., A. Egle, I. Marschitz, M. Kos, G. Bock, H. Ludwig, S. Geley, R. Kofler and R. Greil (1997). "Constitutive expression of Fas (Apo-1/CD95) ligand on multiple myeloma cells: a potential mechanism of tumor-induced suppression of immune surveillance." Blood **90**(1): 12-20.
- Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta and A. Moretta (1998). "NKp44, a novel triggering surface molecule specifically

- expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis." *J Exp Med* **187**(12): 2065-2072.
- Vitale, M., M. Della Chiesa, S. Carlomagno, D. Pende, M. Arico, L. Moretta and A. Moretta (2005). "NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor." *Blood* **106**(2): 566-571.
- Vivier, E. and C. A. Biron (2002). "Immunology. A pathogen receptor on natural killer cells." *Science* **296**(5571): 1248-1249.
- Vivier, E., D. H. Raulet, A. Moretta, M. A. Caligiuri, L. Zitvogel, L. L. Lanier, W. M. Yokoyama and S. Ugolini (2011). "Innate or adaptive immunity? The example of natural killer cells." *Science* **331**(6013): 44-49.
- Vivier, E., E. Tomasello, M. Baratin, T. Walzer and S. Ugolini (2008). "Functions of natural killer cells." *Nat Immunol* **9**(5): 503-510.
- von Lilienfeld-Toal, M., S. Frank, C. Leyendecker, S. Feyler, S. Jarmin, R. Morgan, A. Glasmacher, A. Marten, I. G. Schmidt-Wolf, P. Brossart and G. Cook (2010). "Reduced immune effector cell NKG2D expression and increased levels of soluble NKG2D ligands in multiple myeloma may not be causally linked." *Cancer Immunol Immunother* **59**(6): 829-839.
- Vonderheide, R. H., J. L. Schultze, K. S. Anderson, B. Maecker, M. O. Butler, Z. Xia, M. J. Kuroda, M. S. von Bergwelt-Baildon, M. M. Bedor, K. M. Hoar, D. R. Schnipper, M. W. Brooks, N. L. Letvin, K. F. Stephans, K. W. Wucherpfennig, W. C. Hahn and L. M. Nadler (2001). "Equivalent induction of telomerase-specific cytotoxic T lymphocytes from tumor-bearing patients and healthy individuals." *Cancer Res* **61**(23): 8366-8370.
- Wai, L. E., J. A. Garcia, O. M. Martinez and S. M. Krams (2011). "Distinct roles for the NK cell-activating receptors in mediating interactions with dendritic cells and tumor cells." *J Immunol* **186**(1): 222-229.
- Walzer, T., M. Dalod, S. H. Robbins, L. Zitvogel and E. Vivier (2005). "Natural-killer cells and dendritic cells: 'l'union fait la force'." *Blood* **106**(7): 2252-2258.
- Wang, S., S. Hong, J. Yang, J. Qian, X. Zhang, E. Shpall, L. W. Kwak and Q. Yi (2006). "Optimizing immunotherapy in multiple myeloma: Restoring the function of patients' monocyte-derived dendritic cells by inhibiting p38 or activating MEK/ERK MAPK and neutralizing interleukin-6 in progenitor cells." *Blood* **108**(13): 4071-4077.
- Watson, M. L. (2002). "Chemokines--linking receptors to response." *Immunology* **105**(2): 121-124.
- Weiner, L. M., R. Surana and S. Wang (2010). "Monoclonal antibodies: versatile platforms for cancer immunotherapy." *Nat Rev Immunol* **10**(5): 317-327.
- Wen, X. Y., S. Mandelbaum, Z. H. Li, M. Hitt, F. L. Graham, T. S. Hawley, R. G. Hawley and A. K. Stewart (2001a). "Tricistronic viral vectors co-expressing interleukin-12 (1L-12) and CD80 (B7-1) for the immunotherapy of cancer: preclinical studies in myeloma." *Cancer Gene Ther* **8**(5): 361-370.
- Wen, Y. J., B. Barlogie and Q. Yi (2001b). "Idiotypic-specific cytotoxic T lymphocytes in multiple myeloma: evidence for their capacity to lyse autologous primary tumor cells." *Blood* **97**(6): 1750-1755.
- Wen, Y. J., R. Min, G. Tricot, B. Barlogie and Q. Yi (2002). "Tumor lysate-specific cytotoxic T lymphocytes in multiple myeloma: promising effector cells for immunotherapy." *Blood* **99**(9): 3280-3285.
- Wendtner, C. M., A. Nolte, E. Mangold, R. Buhmann, G. Maass, J. A. Chiorini, E. L. Winnacker, B. Emmerich, R. M. Kotin and M. Hallek (1997). "Gene transfer of the costimulatory molecules B7-1 and B7-2 into human multiple myeloma cells by recombinant adeno-associated virus enhances the cytolytic T cell response." *Gene Ther* **4**(7): 726-735.
- Westermann, J., A. Florcken, G. Willmsky, A. van Lessen, J. Kopp, A. Takvorian, K. Johrens, A. Lukowsky, C. Schonemann, B. Sawitzki, H. Pohla, R. Frank, B. Dorken, D. J. Schendel, T. Blankenstein and A. Pezzutto (2011). "Allogeneic gene-modified tumor cells (RCC-26/IL-7/CD80) as a vaccine in patients with metastatic renal cell cancer: a clinical phase-I study." *Gene Ther* **18**(4): 354-363.
- Wilson, J. L., J. Charo, A. Martin-Fontecha, P. Dellabona, G. Casorati, B. J. Chambers, R. Kiessling, M. T. Bejarano and H. G. Ljunggren (1999). "NK cell triggering by the human costimulatory molecules CD80 and CD86." *J Immunol* **163**(8): 4207-4212.
- Winchester, B. G. (2001). "Lysosomal membrane proteins." *Eur J Paediatr Neurol* **5 Suppl A**: 11-19.
- Yam, P. Y., S. Li, J. Wu, J. Hu, J. A. Zaia and J. K. Yee (2002). "Design of HIV vectors for efficient gene delivery into human hematopoietic cells." *Mol Ther* **5**(4): 479-484.
- Ye, J., J. R. Ortaldo, K. Conlon, R. Winkler-Pickett and H. A. Young (1995). "Cellular and molecular mechanisms of IFN-gamma production induced by IL-2 and IL-12 in a human NK cell line." *J Leukoc Biol* **58**(2): 225-233.

- Yeh, K. Y., B. A. Pulaski, M. L. Woods, A. J. McAdam, A. A. Gaspari, J. G. Frelinger and E. M. Lord (1995). "B7-1 enhances natural killer cell-mediated cytotoxicity and inhibits tumor growth of a poorly immunogenic murine carcinoma." Cell Immunol **165**(2): 217-224.
- Yi, Q., S. Dabadghao, A. Osterborg, S. Bergenbrant and G. Holm (1997). "Myeloma bone marrow plasma cells: evidence for their capacity as antigen-presenting cells." Blood **90**(5): 1960-1967.
- Yokoyama, W. M., S. Kim and A. R. French (2004). "The dynamic life of natural killer cells." Annu Rev Immunol **22**: 405-429.
- Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier and P. Charneau (2000). "HIV-1 genome nuclear import is mediated by a central DNA flap." Cell **101**(2): 173-185.
- Zhang, L., M. Gotz, S. Hofmann and J. Greiner (2012). "Immunogenic targets for specific immunotherapy in multiple myeloma." Clin Dev Immunol **2012**: 820394.
- Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini and D. Trono (1998). "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery." J Virol **72**(12): 9873-9880.